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Synthesis of nigranoic acid and manwuweizic acid derivatives as HDAC inhibitors and anti-inflammatory agents

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ABSTRACT

As a successful anti-tumor drug target, the family of histone deacetylases (HDACs) is also a critical player in immune response, making the research of anti-inflammatory HDAC inhibitors an attractive new focus. In this report, triterpenoids nigranoic acid (NA) and manwuweizic acid (MA) were identified as HDAC inhibitors through docking-based virtual screening and enzymatic activity assay. A series of derivatives of NA and MA were synthesized and assessed for their biological effects. As a result, hydroxamic acid derivatives of NA and MA showed moderately increased activity for HDAC1/2/4/6 inhibition (the lowest IC_{50} against HDAC1 is 1.14 μM), with no activity against HDAC8. In J774A.1 macrophage, compound **1**–**3**, **13** and **17**–**19** demonstrated inhibitory activity against lactate dehydrogenase (LDH) and IL-1β production, without affecting cell viability. Compound **19** increased the histone acetylation level in J774A.1 cells, as well as inhibited IL-1β maturation and caspase-1 cleavage. These results indicated that compound **19** blocks the activation of NLRP3 inflammasome, probably related to HDAC inhibition. This work provided a natural scaffold for developing low-cytotoxic and anti-inflammatory HDAC inhibitors, as well as a class of tool molecules for studying the relationship between HDACs and NLRP3 activation.

1. Introduction

Histone deacetylases (HDACs) are a family of enzymes for removing the acetyl group on acetylated lysines. Besides histones, the substrates of HDAC also include non-histone proteins like tubulin, Hsp90 etc [1]. There are 18 subtypes of HDAC, of which 11 subtypes are Zn^{2+} dependent (HDAC 1–11). HDAC is a successful drug target for cancer therapy (T cell lymphoma, multiple myeloma), and also involved in neurological disorder, infection and inflammation [2]. The potential therapeutic effect of HDAC inhibitors in immune disorders has become a new focus in recent years [3]. However, the relationship between HDAC inhibition and anti-inflammation is complex. Previous studies led to mixed results, depending on the experimental system, pathway under study and dosage of compounds [4]. For instance, with the treatment of HDAC inhibitor SAHA, LPS-induced IL-1β secretion was reduced in human peripheral blood mononuclear cells, but increased in human dendritic cells [5].

Plants belonging to the family Schisandraceae have attracted the attention of pharmacologists and phytochemists, due to their notable medicinal functions. The dried mature fruits of *Schisandra chinensis* and *S. sphenanthera* have been used in traditional Chinese medicine to relieve fatigue, protect the liver, strengthen the heart, reduce blood and treat insomnia for over 2000 years [6]. The chemical components of Schisandraceae often feature lanostane- and cycloartane-type triterpenoids [7]. Nigranoic acid and manwuweizic acid, possessing cycloartane and lanostane skeletons, respectively, are A-ring secocycloartene triterpenoid diacids isolated from several *Schisandra* species [8–12]. These two representative compounds were known with various biological activities, including anti-HIV, anticancer, mental and intellectual functions promotion, antiapoptotic and liver protection [13–21]. In this study, by combining molecular docking-based virtual screening, enzyme inhibition assay, chemical modification and cell-based bioassays, we reported a series of semi-synthesized derivatives of NA and MA as HDAC inhibitors and anti-inflammatory agents.

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2. Results

2.1. Discovery of nigranoic acid as HDAC inhibitor

To identify potential HDAC inhibitors from our in-house natural products library (containing 1068 compounds), virtual screening based on molecular docking was performed using HDAC2 (PDB ID: 4LXZ) as receptor [23] (Fig. 1A). Ten compounds were selected (as described in experiment section) for in vitro inhibitory activity test against HDAC1/2/4/6/8. Under the compound concentration of 100 μM, NA (**1**) was identified as active inhibitor against HDAC1/2/4/6 with the inhibition rate >50%, but not for HDAC8. The interaction mode of **1** with HDAC2 was predicted by molecular docking (Fig. 1B). Compound **1** occupied the same binding pocket of suberoylanilide hydroxamic acid (SAHA), which is the first approved HDAC inhibitor. Tyr308, His146 and Arg275 were involved in the hydrogen binding with **1**. The C-27 carboxyl group of **1**, together with Asp269 and Asp181, were found to form polar interaction with Zn^{2+} in the active center of HDAC2.

2.2. Improved separation of nigranoic acid and manwuweizic acid

Crude fractions containing NA (**1**) and MA (**2**) were isolated from the stem of *Schisandra sphenanthera*, as previously reported [10]. Larger quantities of the natural products, NA (**1**) and MA (**2**), were not able to be separated nor taken to purity. The initial attempt to use the mixture in reaction with *O*-methylhydroxylamine, with the expectation that the desired derivatives (**3** and **4**) might be separated by fractional crystallization. It is interesting that in practice the products cocrystallized thereby making separation of these derivatives also difficult, although good use was made of X-ray diffraction analysis to allow the molecular structures of both to be confirmed (supplementary material). In this case, the crude fractions were used for methylation reaction. Then the products were purified with the silica gel column chromatography to remove the unreacted large polar substances (87% yield). The methylated products **1a** and **2a** can be easily separated by C18 reversed phase chromatographic column in preparative high-performance liquid chromatography (Prep-HPLC) (**1a** yield 44%, **2a** yield 39%). Saponification of the separated esters **1a** and **2a** gave pure samples of the corresponding di-acids **1** (88% yield) and **2** (86% yield) (Scheme 1), whose structures confirmed by comparison of their spectroscopic data with those previously reported [9].

2.3. Synthesis of the derivatives of nigranoic acid and manwuweizic acid

Diamides **3**–**18** derived from **1** and **2** were obtained by the reaction of **1** or **2** with substituted amine or *O*-substituted hydroxylamine in acetonitrile in the presence of triethylamine (TEA) and 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium tetrafluoroborate (TBTU) (Scheme 2). Most of the NMR data appeared as multiple, overlapping signals, consistent with the relevant skeletal frameworks of the two series of derivatives, while expected signals and variations of shifts were observed that corresponded to the various R groups of the relevant amines or hydroxylamines (RH) (Figs. S1–16, supplementary material).

Treatment of **1a** and **2a** with hydroxylamine solution and potassium hydroxide (KOH) in tetrahydrofuran (THF) and methanol afforded the hydroxamic acid derivatives **19** and **20**, respectively (Scheme 3). This method was found to have selectivity in the production of 3-(*N*-hydroxyamido)-27-carboxylic acid derivatives. For *N*-hydroxamido acid formation, regiochemistry derivatives of the ester group at C-3 are commonly produced. Though with excessive hydroxylamine solution, the ester group at C-27, which has a "angelic acid methyl ester group", remains less reactive towards hydroxylamine than the "saturated carboxylic acid methyl ester" at the C-3 position, but susceptible to basic hydrolysis. The molecular formula of compound **19** was established as $C_{30}H_{47}NO_4$, on the basis of the high resolution (HR)-electrospray ionisation (ESI)-MS analysis which showed an adduct ion $[M+Na]^+$ at *m/z* 508.3397 (Calcd 508.3397), indicating only one side *N*-hydroxamido acid formation. Comparison of the ^{13}C NMR of compound **19** and NA showed a considerable upfield shift of the C-3 signal in the former. In contrast, the signal position of the C-27 in the ^{13}C NMR spectra of compound **19** closely resembled that in the NA ^{13}C NMR spectrum. This gave good evidence of specific introduction of the hydroxamic acidification at position C-3. The same method proved the *N*-hydroxamido acid formation of compound **20** occurred at position C-3 (Figs. S19–20,

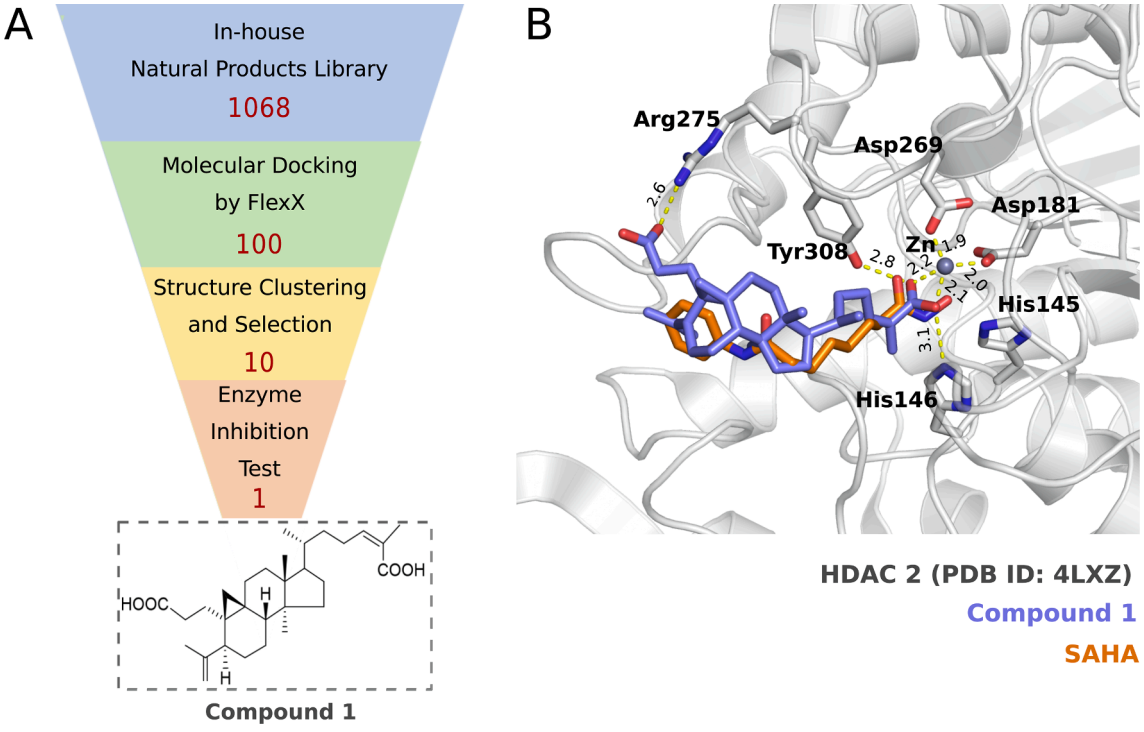
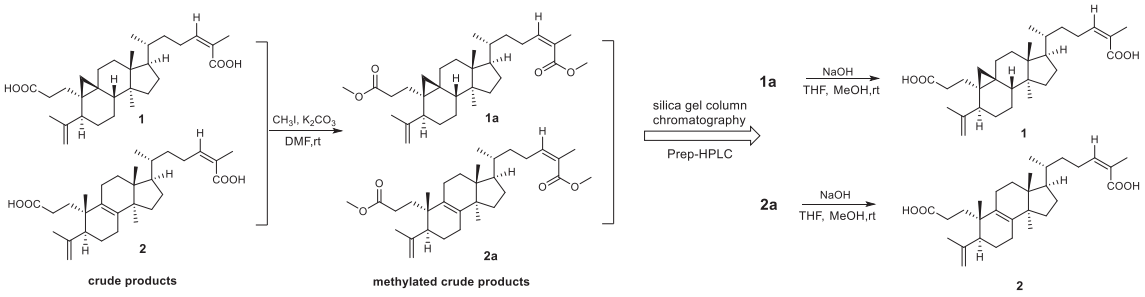
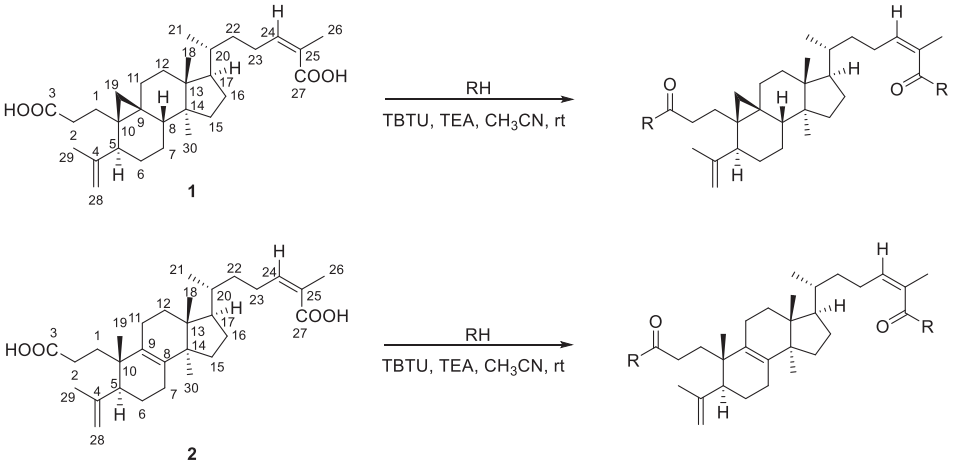


Fig. 1. Virtual screening of HDAC inhibitors from in-house natural products library.



Scheme 1. Improved Separation of Compounds **1** and **2.**



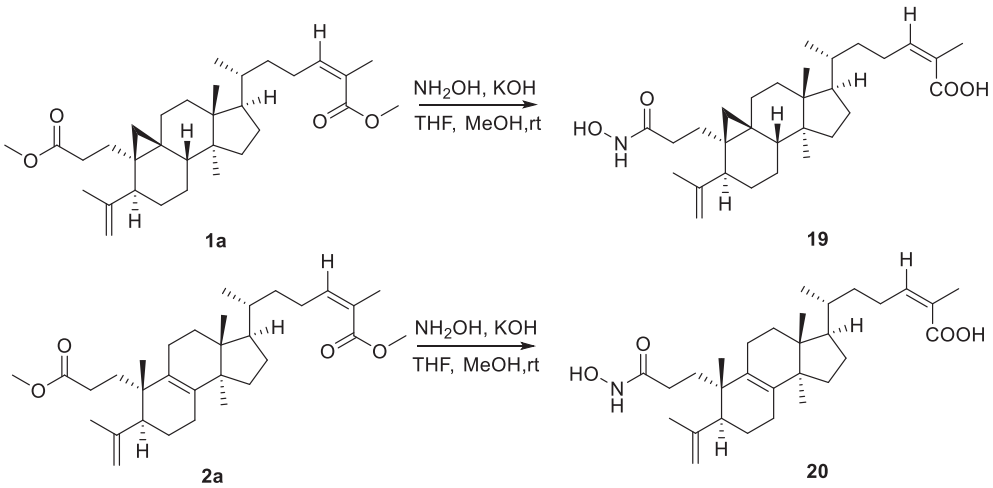
compounds	acids	R	yields	compounds	acids	R	yields
3	**1**		53%	**11**	**1**		71%
4	**2**		49%	**12**	**2**		57%
5	**1**		82%	**13**	**1**		76%
6	**2**		71%	**14**	**2**		66%
7	**1**		54%	**15**	**1**		53%
8	**2**		57%	**16**	**2**		63%
9	**1**		67%	**17**	**1**		52%
10	**2**		56%	**18**	**2**		55%

Scheme 2. General Synthesis of Compounds **3**–**18**.

supplementary material).

2.4. HDAC inhibitory activity of the derivatives of nigranoic acid and manwuweizic acid

Compared to **1** and **2**, **19** and **20** possessed moderately increased



Scheme 3. General Synthesis of Compounds **19**–**20**.

HDAC inhibitory activity, whereas other compounds have not shown significant activity (IC_{50} > 50 μM) (Table 1). The implementation of hydroxamic acid group, the most widely used zinc-biding group in HDAC inhibitors, improved the interaction towards the zinc ion in HDACs. (Fig. 2). The hydroxamate moiety of **19** formed zinc chelation together with the histidine and aspartic acid residues in the catalytic center of HDAC1/2/4/6. The shortest distance between the hydroxamate and Zn^{2+} is 1.9 Å for HDAC1, 1.8 Å for HDAC2, 2.3 Å for HDAC4 and 2.1 Å for HDAC6. The triterpenoid skeleton acted as the hydrophobic linker in HDAC inhibitors, fitted into the neck of HDAC pocket. An extra hydrogen bond was formed by the carboxyl group in **19** with a phenylalanine in HDAC1 (Phe205), HDAC4 (Phe168), HDAC6 (Phe680), but not in HDAC2. It is common for plant-derived HDAC inhibitors to possess a micromolar IC_{50} against HDACs[1]. Although weaker than SAHA in HDAC inhibition, these compounds may still be valuable as low-cytotoxicity HDAC inhibitors, for developing anti-inflammatory agents.

2.5. Anti-inflammatory activity of the derivatives of nigranoic acid and manwuweizic acid

Compounds **1**–**20** were tested for their anti-inflammatory activity in murine macrophage J774A.1. summarized in Table 2, compounds **1**–**3**, **13**, **17**–**19** exhibited inhibitory activity against the production of LDH and IL-1β, without reducing the cell viability. However, compound **20**, although possessing similar structure with **19**, was cytotoxic towards J774A.1 cells. The IC_{50} of LDH and IL-1β production of other compounds were above 20 μM (data not shown). In western blot assays, compound **19** was observed to inhibit IL-1β (Fig. 3A–B) maturation and caspase-1 cleavage (Fig. 3D–E).

To verify their HDAC inhibitory effects in cell level, we analyzed the acetylated-histone level of macrophages treated by compound **19**. Total histone fraction from J774A.1 cells was isolated and then performed by a western blot assay. As shown in Fig. 3C and F, compound **19** increased the histone acetylation level in a dose-dependent manner, indicating that compound **19** inhibits HDAC activity in cell level, which was consistent with the enzyme inhibition assay above. These results suggested that compound **19** blocks NLRP3 inflammasome activation, probably due to the inhibitory effects against HDAC.

Table 1
HDAC inhibitory activity.

Compound	IC_{50} (μM)				
	HDAC1	HDAC2	HDAC4	HDAC6	HDAC8
1	3.96	11.53	>50	3.35	>50
2	8.63	15.74	>50	6.31	>50
19	1.14	10.56	19.39	2.23	>50
20	2.64	3.56	>50	1.08	>50
SAHA[a]	0.020	0.025	–	0.014	0.210
TMP269[b]	–	–	0.289	–	–

[a] SAHA was used as positive control in assays for HDAC1,2,6 and 8.
[b] TMP269 was used as positive control in the assay for HDAC4.

3. Conclusion

In this work, we reported a series of semi-synthesized derivatives of NA and MA, possessing HDAC inhibitory and anti-inflammatory activity. The most potent compound **19** was identified to inhibit HDAC1/2/4/6, but not HDAC8. The IC_{50} of compound **19** against HDACs ranges from 1.139 to 10.558 μM, weaker than the reference compounds SAHA and TMP269. At the same time, compared to the anti-tumor HDAC inhibitors, **19** is low-cytotoxic against J774A.1 (CC_{50} > 20 μM), making it potential for development of anti-inflammatory agent. Treatment with compound **19** was found to increase the histone acetylation level in LPS + nigericin co-stimulated J774A.1 cells, as well as to reduce the expression of IL-1β and caspase-1. These results indicated that compound **19** inhibited the activity of HDACs and activation of NLRP3 inflammasome in inflammation cell model. However, further study is expected to reveal the specific relationship between the inhibition of HDAC and NLRP3 inflammasome activation.

4. Experimental section

4.1. Chemistry materials and methods

Nigranoic acid (NA, **1**) and manwuweizic acid (MA, **2**) were isolated as a mixture as previously described [11]. Other chemicals and solvents used in synthesis were purchased from commercial supplies and were further purified or dried when necessary. All reactions were monitored by analytical thin layer chromatography (TLC) on silica gel pates GF 254 (Qingdao Haiyang Chemical, China). Column chromatography was performed with silica gel (200–300 mesh). Preparative HPLC was carried out on an Agilent 1260 Chromatograph equipped with a diode array detector with a semi-preparative Agilent ZORBAX Extend-C18 reversed-phase column (9.4 × 250 mm, 5 μm). All solvents used in HPLC were of chromatographic grade (Fisher Scientific, NJ, USA). ^{1}H and ^{13}C NMR spectra were recorded on a Bruker AM-400 MHz spectrometer using $CDCl_3$, Pyridine-d_5 or Methanol-d_4 as solvents (TMS as internal standard), and the chemical shifts were reported in δ (ppm) and the coupling constants (J) in Hz. HRESIMS data were obtained on an Agilent LC/MSD

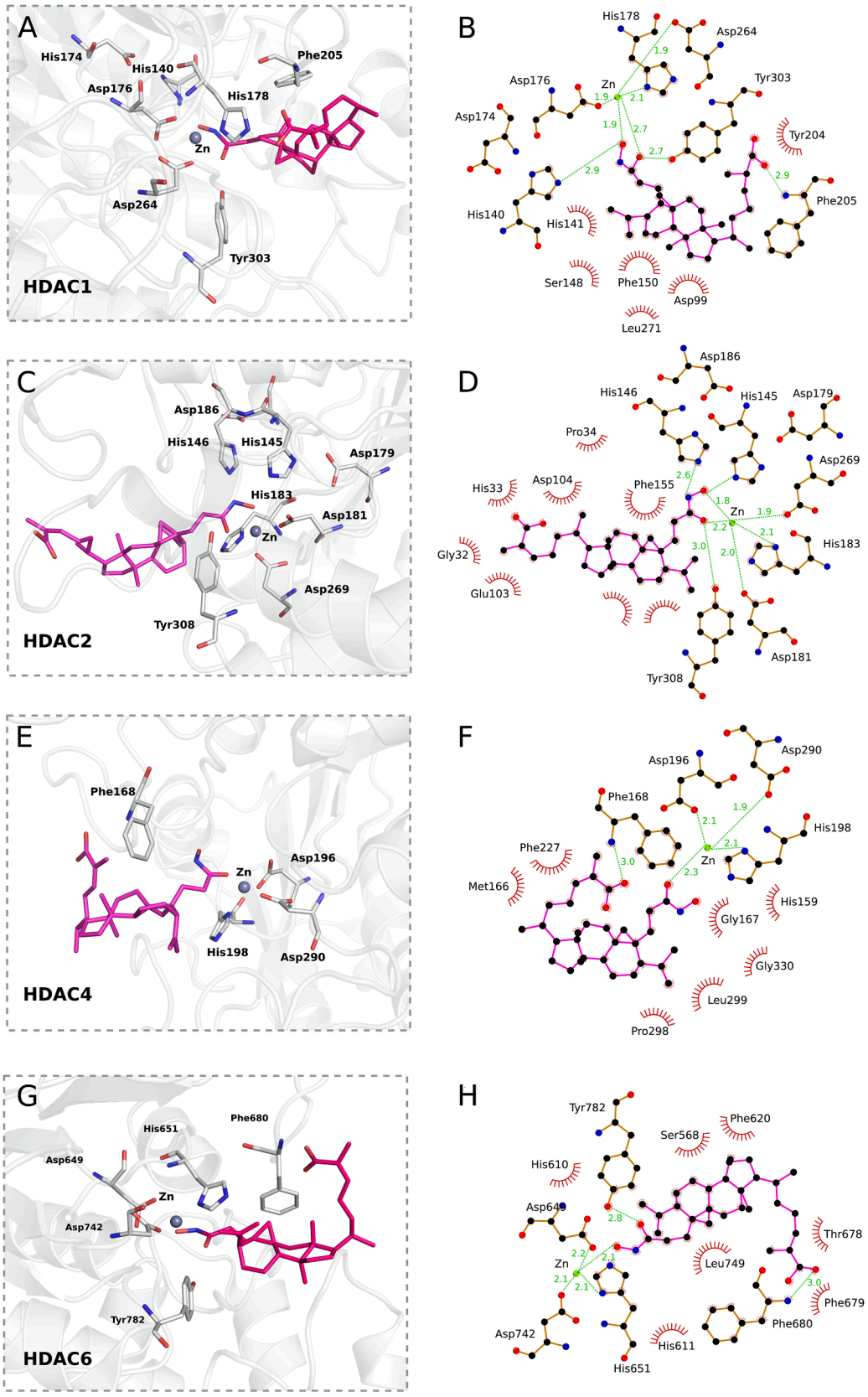


Fig. 2. Binding modes of Compound **19** (in magenta sticks) in HDAC1 (A, B), HDAC2 (C, D), HDAC4 (E, F) and HDAC6 (G, H). Hydrogen bonds are shown in dash line. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 2
Anti-inflammatory effect of compounds in J774A.1 cells.

Compound	IC_{50} mean ± SD (μM,n = 3[b])		CC_{50} mean ± SD (μM,n = 3)
	LDH	IL-1β	
1	3.57 ± 0.41	4.09 ± 0.51	>20
2	7.48 ± 0.88	9.32 ± 1.43	>20
3	6.32 ± 0.91	8.96 ± 1.36	>20
13	5.97 ± 0.98	3.01 ± 0.41	>20
17	7.42 ± 0.84	2.5 ± 0.22	>20
18	15.98 ± 1.33	14.88 ± 1.32	>20
19	9.98 ± 1.01	5.50 ± 0.51	>20
20	>2.50	>2.50	2.90 ± 0.60
SAHA	>2.50	>2.50	2.50 ± 0.80
MCC950[a]	0.31 ± 0.01	0.51 ± 0.02	>20

[a] Positive control.
[b] The experiment was repeated three times.

TOF mass spectrometer. The IR spectra were recorded using a Thermo Scientific Nicolet iS10 spectrometer. Melting points were determined on a WRS-1 digital melting point instrument (Shanghai, China).

4.1.1. Procedures for the preparation of Di-esters 1a and 2a

To a solution of crude fractions (**1** and **2**, 1.60 g, 3.41 mmol) in DMF (25 mL) was added K_2CO_3 (1.89 g, 13.6 mmol) and stirred for 1.5 h at room temperature. To the mixture added CH_3I (1.44 g, 10.2 mmol) and stirred at room temperature was continued for 4 h. After the reaction was complete, the reaction mixture was diluted with H_2O (150 mL) and extracted with EtOAc (3 × 60 mL). The combined organic layers were washed with brine (100 mL). The organic phase was dried over anhydrous Na_2SO_4. Filtration and evaporation of the solvent at reduced pressure gave a crude product, which was column chromatographed on silica gel (petroleum ether-EtOAc, 5:1) and the combined, less polar fractions (1.48 g, 87% yield) subjected to preparative HPLC with CH_3CN-H_2O (95:5) (5 mL/min) to give separated di-esters **1a** and **2a** as oils. Some of them were used to get corresponding di-acids **1** and **2** through saponification reaction, and the rest to synthesize compounds **19** and **20**.

Dimethyl 3,4-secocycloarta-4(28),24-(Z)-diene-3,-27-dioate ***(1a).*** colourless oil. 755 mg, 44% yield; IR ν_{max} (KBr) 2962, 1741, 1721, 1644, 1435, 1375, 1261, 1197, 1095, 1028, 866, 800 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 5.92 (1H, t, J = 7.2 Hz, H-24), 4.80 (1H, s, H_A-28), 4.72 (1H, s, H_B-28), 3.73, 3.64 (6H, s, $OCH3_{(A)}$ and $OCH3_{(B)}$), 1.88, 1.67 (6H, s, CH_3-26, CH_3-29), 2.55–1.04 (24H, m, nigranoic acid scaffold), 0.95, 0.92 (6H, s, CH_3-18, CH_3-30), 0.89 (3H, d, J = 6.4 Hz, CH_3-21), 0.72 (1H, d, J = 4.6 Hz, H_β-19), 0.40 (1H, d, J = 4.4 Hz, H_α-19); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 174.5 (C, C-3), 168.7 (C, C-27), 149.6 (C, C-4), 144.3 (CH, C-24), 126.6 (C, C-25), 111.6 (CH_2, C-28), 52.3 (CH, C-17), 51.6 ($OC_{(A)}H_3$), 51.3 ($OC_{(B)}H_3$), 49.1 (C, C-14), 47.8 (CH, C-8), 46.0 (CH, C-5), 45.3 (C, C-13), 36.1 (CH, C-20), 36.0 (CH_2, C-11), 35.8 (CH_2, C-12), 33.2 (CH_2, C-15), 31.6 (CH_2, C-2), 30.1 (CH_2, C-19), 29.2 (CH_2, C-1), 28.2 (CH_2, C-16), 27.9 (CH_2, C-6), 27.2 (C, C-10), 27.1 (CH_2, C-22), 26.8 (CH_2, C-23), 25.1 (CH_2, C-7), 21.5 (C, C-9), 20.8 (CH_3, C-26), 19.9 (CH_3, C-29), 19.4 (CH_3, C-30), 18.3 (CH_3, C-21), 18.2 (CH_3, C-18); HRMS-ESI: *m/z* calcd for $C_{32}H_{50}NaO_4$ $(M+Na)^+$ 521.3601, found 521.3599.

Dimethyl 3,4-secocycloarta-4(28),8,24-(Z)-diene-3,-27-dioate ***(2a).*** colourless oil. 664 mg, 39% yield; IR ν_{max} (KBr) 2963, 1746, 1714, 1681, 1646, 1571, 1415, 1261, 1093, 1025, 800 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 5.93 (1H, t, J = 7.4 Hz, H-24), 4.89 (1H, s, H_A-28), 4.67 (1H, s, H_B-28), 3.73, 3.65 (6H, s, $OCH3_{(A)}$ and $OCH3_{(B)}$), 1.89, 1.75 (6H, s, CH_3-26, CH_3-29), 2.55–1.08 (23H, m, manwuweizic acid scaffold), 0.95 (3H, s, CH_3-18), 0.94–0.89 (6H, overlap, CH_3-30, CH_3-21), 0.73 (3H, s, CH_3-19); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 174.8 (C, C-3), 168.6 (C, C-27), 147.4 (C, C-4), 144.1 (CH, C-24), 139.1 (C, C-9), 129.4 (C, C-8), 126.4 (C, C-25), 113.8 (CH_2, C-28), 51.5 ($OC_{(A)}H_3$), 51.2 ($OC_{(B)}H_3$), 50.8 (C, C-14), 50.3 (CH, C-17), 46.8 (CH, C-5), 44.4 (C, C-13), 40.3 (C, C-10), 36.4 (CH,

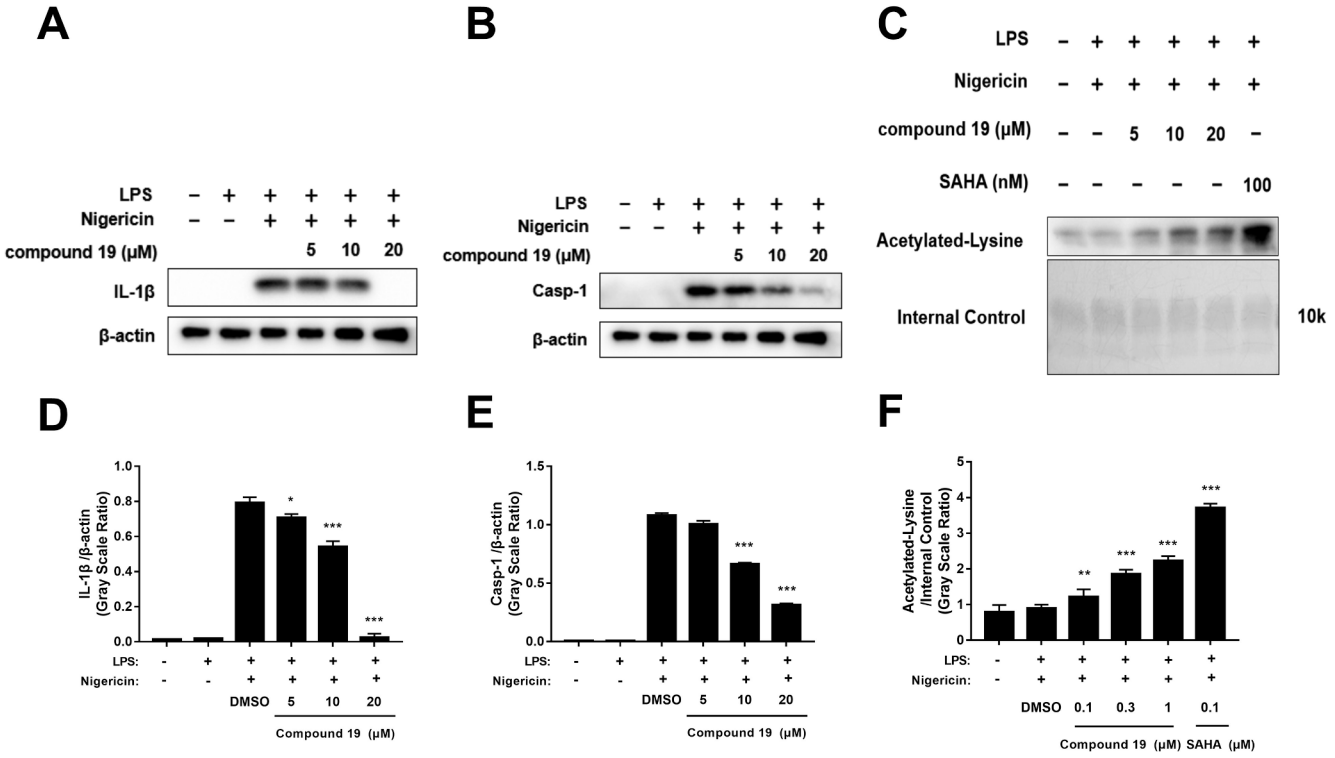


Fig. 3. Compound **19** inhibited the activity of histone deacetylas and blocked NLRP3 inflammasome activation. (A) IL-1β expression in LPS + nigericin co-stimulated J774A.1 cultured supernatant treated with Compound **19**. (B) Caspase-1 expression in LPS + nigericin co-stimulated J774A.1 cultured supernatant treated with Compound **19**. (C) Histone acetylation levels in LPS + nigericin co-stimulated J774A.1 cultured supernatant treated with Compound **19**. SAHA (100 nM) was used as a positive control. (D)–(F) Gray analysis results. Data represent the mean values of three experiments (±SD). *P < 0.05,**P < 0.01, ***P < 0.001.

C-20), 35.9 (CH_2, C-1), 32.5 (CH_2, C-2), 31.1 (CH_2, C-6), 31.0 (CH_2, C-7), 29.4 (CH_2, C-11), 28.0 (CH_2, C-12), 26.7 (CH_2, C-15), 25.9 (CH_2, C-16), 25.2 (CH_3, C-30), 24.0 (CH_2, C-22), 23.0 (CH_3, C-19), 22.3 (CH_3, C-18), 21.7 (CH_2, C-23), 20.7 (CH_3, C-21), 18.6 (CH_3, C-26), 15.9 (CH_3, C-29); HRMS-ESI: *m/z* calcd for $C_{32}H_{50}NaO_4$ $(M+Na)^+$ 521.3601, found 521.3597.

*4.1.2. Procedures for the synthesis of NA **(1)** and MA **(2)***

2 N aqueous NaOH (6 mL, 12 mmol) was added to a solution of di-ester **1a** (667 mg, 1.34 mmol) in THF-MeOH (1:1, 20 mL). The mixture was stirred at room temperature for 6 h, acidified to pH 3 with 1 N HCl solution and extracted with EtOAc (3 × 50 mL). The combined organic layers were washed with brine (80 mL) and dried over anhydrous Na_2SO_4. Evaporation of the solvent under reduced pressure and column chromatography of the residue (CH_2Cl_2-MeOH, 15:1) gave NA (**1**) (552 mg, 88% yield). Treatment of di-ester **2a** (542 mg, 1.09 mmol) under the same conditions and scale gave MA (**2**) (442 mg, 86% yield).

*4.1.3. Procedures for the synthesis of compound **3**–**18***

To a solution of acid (**1** or **2**, 47 mg, 0.10 mmol) and TBTU (71 mg, 0.22 mmol) in anhydrous CH_3CN (1 mL) was added TEA (40 mg, 0.40 mmol) and the mixture stirred for 30 min at room temperature. To the mixture added substituted amine or *O*-substituted hydroxylamine (0.23 mmol) and stirred at room temperature was continued for 2 h. After the reaction was complete, the reaction mixture was diluted with H_2O (15 mL) and extracted with DCM (3 × 10 mL). The combined organic layers were washed with brine (20 mL). The organic phase was dried over anhydrous Na_2SO_4. Filtration and evaporation of the solvent at reduced pressure gave a crude product, which was purified by column chromatography (petroleum ether-EtOAc, 3:2) to give the pure corresponding diamide (**3**–**18**).

*N,N'-dimethoxy 3,4-secocycloarta-4(28),24-(Z)-diene-3,-27-diamide **(3)***. According to the above general procedures, derivative **3** was prepared via synthesis of NA (**1**) with *O*-methylhydroxylamine hydrochloride; white solid; 28 mg, 53% yield; mp 104–107 °C; IR ν_{max} (KBr) 3443, 2924, 2854, 1643, 1457, 1383, 1262, 1076, 1053 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 8.31, 8.25 (2H, s, $CONH_{(A)}$ and $CONH_{(B)}$), 5.58 (1H, t, J = 7.2 Hz, H-24), 4.81 (1H, s, H_A-28), 4.73 (1H, s, H_B-28), 3.82 (3H, s, $OCH_{3(A)}$), 3.73 (3H, s, $OCH_{3(B)}$), 1.88, 1.67 (6H, s, CH_3-26, CH_3-29), 2.50–1.04 (24H, m, nigranoic acid scaffold), 0.94, 0.91 (6H, s, CH_3-18, CH_3-30), 0.87 (3H, d, J = 6.4 Hz, CH_3-21), 0.73 (1H, d, J = 4.5 Hz, H_β-19), 0.40 (1H, d, J = 4.5 Hz, H_α-19); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 172.8 (C, C-3), 168.1 (C, C-27), 149.8 (C, C-4), 136.1 (CH, C-24), 128.5 (C, C-25), 111.5 (CH_2, C-28), 64.6 ($OC_{(A)}H_3$), 64.5 ($OC_{(B)}H_3$), 52.0 (CH, C-17), 48.9 (C, C-14), 47.4 (CH, C-8), 46.1 (CH, C-5), 45.1 (C, C-13), 36.1 (CH_2, C-11), 35.9 (CH, C-20), 35.5 (CH_2, C-12), 33.0 (CH_2, C-15), 29.7 (CH_2, C-2), 29.6 (CH_2, C-19), 29.3 (CH_2, C-1), 28.0 (CH_2, C-16), 27.6 (CH_2, C-6), 27.1 (C, C-10), 27.9 (CH_2, C-22), 26.5 (CH_2, C-23), 24.9 (CH_2, C-7), 21.3 (C, C-9), 20.5 (CH_3, C-26), 19.7 (CH_3, C-29), 19.3 (CH_3, C-30), 18.1 (CH_3, C-21), 17.9 (CH_3, C-18); HRMS-ESI: *m/z* calcd for $C_{32}H_{52}N_2NaO_4$ $(M+Na)^+$ 551.3819, found 551.3817.

*N,N'-dimethoxy 3,4-secolanosta-4(28),8,24-(Z)-trien-3,-27-diamide **(4)***. According to the above general procedures, derivative **4** was prepared via synthesis of MA (**2**) with *O*-methylhydroxylamine hydrochloride; white solid; 26 mg, 49% yield; mp 124–125 °C; IR ν_{max} (KBr) 3440, 2923, 2853, 1640, 1402, 1323, 1263, 1038 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 8.66, 844 (2H, s, $CONH_{(A)}$ and $CONH_{(B)}$), 5.58 (1H, t, J = 7.1 Hz, H-24), 4.88 (1H, s, H_A-28), 4.68 (1H, s, H_B-28), 3.80 (3H, s, $OCH_{3(A)}$), 3.73 (3H, s, $OCH_{3(B)}$), 1.87, 1.75 (6H, s, CH_3-26, CH_3-29), 2.33–1.01 (23H, m, manwuweizic acid scaffold), 0.94 (3H, s, CH_3-18), 0.92–0.79 (6H, overlap, CH_3-30, CH_3-21), 0.72 (3H, s, CH_3-19); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 171.7 (C, C-3), 168.2 (C, C-27), 147.4 (C, C-4), 139.1 (C, C-9), 136.0 (CH, C-24), 129.6 (C, C-8), 128.4 (C, C-25), 113.8 (CH_2, C-28), 64.5 ($OC_{(A)}H_3$), 64.5 ($OC_{(B)}H_3$), 50.8 (C, C-14), 50.2 (CH, C-17), 47.0 (CH, C-5), 44.4 (C, C-13), 40.5 (C, C-10), 36.3 (CH, C-20), 36.2 (CH_2, C-11), 36.2 (CH_2, C-1), 31.1 (CH_2, C-2), 31.1 (CH_2, C-6), 31.0 (CH_2, C-7), 28.1 (CH_2, C-12), 26.5 (CH_2, C-15), 25.9 (CH_2, C-16), 25.2 (CH_3, C-30), 24.0 (CH_2, C-22), 23.0 (CH_3, C-19), 22.3 (CH_3, C-18), 21.8 (CH_2, C-23), 20.5 (CH_3, C-21), 18.6 (CH_3, C-26), 16.0 (CH_3, C-29); HRMS-ESI: *m/z* calcd for $C_{32}H_{52}N_2NaO_4$ $(M+Na)^+$ 551.3819, found 551.3820.

*N,N'-diallyl 3,4-secocycloarta-4(28),24-(Z)-diene-3,-27-diamide **(5)***. According to the above general procedures, derivative **5** was prepared via synthesis of NA (**1**) with allylamine hydrochloride; white solid; 45 mg, 82% yield; mp 132–134 °C; IR ν_{max} (KBr) 3439, 3309, 2924, 2878, 1652, 1617, 1531, 1382, 1146, 999 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 5.91–5.72 (2H, overlap, $CH_{(A)}$═CH_2 and $CH_{(B)}$═CH_2), 5.72–5.58 (2H, overlap, $CONH_{(A)}$ and $CONH_{(B)}$), 5.49 (1H, t, J = 7.5 Hz, H-24), 5.26–5.03 (4H, overlap, CH═$CH_{2(A)}$ and CH═$CH_{2(B)}$), 4.79 (1H, s, H_A-28), 4.71 (1H, s, H_B-28), 3.98–3.86 (2H, m, $CH_{2(A)}$—CH═CH_2), 3.86–3.75 (2H, m, $CH_{2(B)}$–CH═CH_2), 1.88, 1.66 (6H, s, CH_3-26, CH_3-29), 2.46–1.02 (24H, m, nigranoic acid scaffold), 0.92, 0.89 (6H, s, CH_3-18, CH_3-30), 0.84 (3H, d, J = 6.4 Hz, CH_3-21), 0.70 (1H, d, J = 4.5 Hz, H_β-19), 0.37 (1H, d, J = 4.5 Hz, H_α-19); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 173.0 (C, C-3), 170.0 (C, C-27), 149.8 (C, C-4), 134.3 (CH, C-24), 134.2 ($C_{(A)}H$═CH_2), 134.1 ($C_{(B)}H$═CH_2), 131.4 (C, C-25), 116.5 (CH═$C_{(A)}H_2$), 116.1 (CH═$C_{(A)}H_2$), 111.4 (CH_2, C-28), 52.0 (CH, C-17), 49.0 (C, C-14), 47.4 (CH, C-8), 45.9 (CH, C-5), 45.1 (C, C-13), 41.8 ($C_{(A)}H_2$-CH═CH_2), 41.7 ($C_{(B)}H_2$-CH═CH_2), 36.2 (CH_2, C-11), 35.8 (CH, C-20), 35.5 (CH_2, C-12), 33.8 (CH_2, C-15), 32.9 (CH_2, C-2), 29.9 (CH_2, C-19), 29.7 (CH_2, C-1), 28.0 (CH_2, C-16), 27.6 (CH_2, C-6), 27.1 (C, C-10), 26.9 (CH_2, C-22), 26.3 (CH_2, C-23), 24.9 (CH_2, C-7), 21.3 (C, C-9), 20.9 (CH_3, C-26), 19.6 (CH_3, C-29), 19.2 (CH_3, C-30), 18.1 (CH_3, C-21), 17.9 (CH_3, C-18); HRMS-ESI: *m/z* calcd for $C_{36}H_{56}N_2NaO_2$ $(M+Na)^+$ 571.4234, found 571.4238.

*N,N'-diallyl 3,4-secolanosta-4(28),8,24-(Z)-trien-3,-27-diamide **(6)***. According to the above general procedures, derivative **6** was prepared via synthesis of MA (**2**) with allylamine hydrochloride; white solid; 39 mg, 71% yield; mp 123–125 °C; IR ν_{max} (KBr) 3432, 2925, 1640, 1546, 1401, 1323, 1267 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 5.91–5.74 (2H, overlap, $CH_{(A)}$═CH_2 and $CH_{(B)}$═CH_2), 5.73–5.63 (2H, overlap, $CONH_{(A)}$ and $CONH_{(B)}$), 5.48 (1H, t, J = 7.4 Hz, H-24), 5.23–5.05 (4H, overlap, CH═$CH_{2(A)}$ and CH═$CH_{2(B)}$), 4.87 (1H, s, H_A-28), 4.66 (1H, s, H_B-28), 3.98–3.86 (2H, m, $CH_{2(A)}$—CH═CH_2), 3.86–3.78 (2H, m, $CH_{2(B)}$—CH═CH_2), 1.87, 1.73 (6H, s, CH_3-26, CH_3-29), 2.30–1.01 (23H, m, manwuweizic acid scaffold), 0.93 (3H, s, CH_3-18), 0.91–0.84 (6H, overlap, CH_3-30, CH_3-21), 0.69 (3H, s, CH_3-19); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 173.3 (C, C-3), 170.0 (C, C-27), 147.5 (C, C-4), 138.7 (C, C-9), 134.4 (CH, C-24), 134.2 ($C_{(A)}H$═CH_2), 134.1 ($C_{(B)}H$═CH_2), 131.4 (C, C-8), 129.5 (C, C-25), 116.5 (CH═$C_{(A)}H_2$), 116.2 (CH═$C_{(B)}H_2$), 113.6 (CH_2, C-28), 50.7 (C, C-14), 50.2 (CH, C-17), 46.9 (CH, C-5), 44.3 (C, C-13), 41.8 ($C_{(A)}H_2$-CH═CH_2), 41.7 ($C_{(B)}H_2$-CH═CH_2), 40.3 (C, C-10), 36.2 (CH_2, C-1), 36.2 (CH, C-20), 33.2 (CH_2, C-2), 31.7 (CH_2, C-6), 31.0 (CH_2, C-7), 30.9 (CH_2, C-11), 28.0 (CH_2, C-12), 26.4 (CH_2, C-15), 25.8 (CH_2, C-16), 25.2 (CH_3, C-30), 23.9 (CH_2, C-22), 22.9 (CH_3, C-19), 22.3 (CH_3, C-18), 21.7 (CH_2, C-23), 20.9 (CH_3, C-21), 18.5 (CH_3, C-26), 15.9 (CH_3, C-29); HRMS-ESI: *m/z* calcd for $C_{36}H_{57}N_2O_2$ $(M+H)^+$ 549.4415, found 549.4413.

*N,N'-diethoxy 3,4-secocycloarta-4(28),24-(Z)-diene-3,-27-diamide **(7)***. According to the above general procedures, derivative **7** was prepared via synthesis of NA (**1**) with ethoxyamine hydrochloride; white solid; 30 mg, 54% yield; mp 93–98 °C; IR ν_{max} (KBr) 3440, 2924, 2854, 1640, 1495, 1458, 1402, 1324, 1263, 1039 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 8.30, 8.23 (2H, s, $CONH_{(A)}$ and $CONH_{(B)}$), 5.56 (1H, t, J = 7.2 Hz, H-24), 4.81 (1H, s, H_A-28), 4.72 (1H, s, H_B-28), 4.17–3.97 (2H, m, J = 6.4 Hz, $OCH_{2(A)}CH_3$), 3.96–3.77 (2H, m, J = 6.3 Hz, $OCH_{2(B)}CH_3$), 1.87, 1.67 (6H, s, CH_3-26, CH_3-29), 2.49–1.04 (30H, m, nigranoic acid scaffold, $OCH_2CH_{3(A)}$, $OCH_2CH_{3(B)}$), 0.93, 0.90 (6H, s, CH_3-18, CH_3-30), 0.86 (3H, d, J = 6.4 Hz, CH_3-21), 0.72 (1H, d, J = 4.5 Hz, H_β-19), 0.40 (1H, d, J = 4.5 Hz, H_α-19); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 171.4 (C, C-3), 168.3 (C, C-27), 149.8 (C, C-4), 135.8 (CH, C-24), 128.5 (C, C-25), 111.4 (CH_2, C-28), 72.3 ($OC_{(A)}H_2CH_3$), 72.1 ($OC_{(B)}H_2CH_3$), 52.0 (CH, C-17),

48.9 (C, C-14), 47.5 (CH, C-8), 46.1 (CH, C-5), 45.1 (C, C-13), 36.1 (CH_2, C-11), 35.9 (CH, C-20), 35.5 (CH_2, C-12), 33.0 (CH_2, C-15), 29.7 (CH_2, C-2), 29.7 (CH_2, C-19), 29.7 (CH_2, C-1), 28.0 (CH_2, C-16), 27.7 (CH_2, C-6), 27.2 (C, C-10), 27.0 (CH_2, C-22), 26.5 (CH_2, C-23), 24.9 (CH_2, C-7), 21.3 (C, C-9), 20.6 (CH_3, C-26), 19.7 (CH_3, C-29), 19.2 (CH_3, C-30), 18.1 (CH_3, C-21), 17.9 (CH_3, C-18), 13.5($OCH_2C_{(A)}H3$), 13.4($OCH_2C_{(B)}H_3$); HRMS-ESI: *m/z* calcd for $C_{34}H_{56}N_2NaO_4$ $(M+Na)^+$ 579.4132, found 579.4129.

N,N'-diethoxy 3,4-secolanosta-4(28),8,24-(Z)-trien-3,-27-diamide ***(8)***. According to the above general procedures, derivative **8** was prepared via synthesis of MA (**2**) with ethoxyamine hydrochloride; white solid; 32 mg, 57% yield; mp 115–120 °C; IR ν_{max} (KBr) 3445, 2924, 1640, 1401, 1052 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 8.35, 8.23 (2H, s, $CONH_{(A)}$ and $CONH_{(B)}$), 5.55 (1H, t, $J = 7.2$ Hz, H-24), 4.88 (1H, s, H_A-28), 4.68 (1H, s, H_B-28), 4.09–3.79 (4H, overlap, $OCH_{2(A)}CH_3$ and $OCH_{2(B)}CH_3$), 1.87, 1.75 (6H, s, CH_3-26, CH_3-29), 1.28 (3H, t, $J = 6.8$ Hz, $OCH_2CH_{3(A)}$), 1.24 (3H, t, $J = 7.0$ Hz, $OCH_2CH_{3(B)}$), 2.33–1.05 (23H, m, manwuweizic acid scaffold), 0.94 (3H, s, CH_3-18), 0.92–0.86 (6H, overlap, CH_3-30, CH_3-21), 0.71 (3H, s, CH_3-19); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 171.6 (C, C-3), 168.3 (C, C-27), 147.4 (C, C-4), 139.0 (C, C-9), 135.8 (CH, C-24), 129.5 (C, C-8), 128.6 (C, C-25), 113.7 (CH_2, C-28), 72.3 ($OC_{(A)}H_2CH_3$), 72.1 ($OC_{(B)}H_2CH_3$), 50.7 (C, C-14), 50.2 (CH, C-17), 47.0 (CH, C-5), 44.3 (C, C-13), 40.4 (C, C-10), 36.2 (CH, C-20), 36.1 (CH_2, C-11), 36.0 (CH_2, C-1) 31.1 (CH_2, C-2), 31.1 (CH_2, C-6), 30.9 (CH_2, C-7), 28.0 (CH_2, C-12), 26.5 (CH_2, C-15), 25.8 (CH_2, C-16), 25.2 (CH_3, C-30), 24.0 (CH_2, C-22), 22.9 (CH_3, C-19), 22.3 (CH_3, C-18), 21.7 (CH_2, C-23), 20.6 (CH_3, C-21), 18.5 (CH_3, C-26), 15.9 (CH_3, C-29); HRMS-ESI: *m/z* calcd for $C_{34}H_{56}N_2NaO_4$ $(M+Na)^+$ 579.4132, found 579.4132.

N,N'-dipropyl 3,4-secocycloarta-4(28),24-(Z)-diene-3,-27-diamide ***(9)***. According to the above general procedures, derivative **9** was prepared via synthesis of NA (**1**) with 1-propanamine; white solid; 37 mg, 67% yield; mp 114–118 °C; IR ν_{max} (KBr) 3442, 2923, 2853, 1652, 1545, 1457, 1382, 1233, 1151 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 5.53–5.30 (3H, overlap, $CONH_{(A)}$, $CONH_{(B)}$, H-24), 4.81 (1H, s, H_A-28), 4.73 (1H, s, H_B-28), 3.35–3.21 (2H, m, $J = 6.8$, 2.6 Hz, $CH_{2(A)}CH_2CH_3$), 3.21–3.11 (2H, m, $J = 6.6$ Hz, $CH_{2(B)}CH_2CH_3$), 1.88, 1.68 (6H, s, CH_3-26, CH_3-29), 2.49–1.04 (28H, m, nigranoic acid scaffold, $CH_2CH_{2(A)}CH_3$, $CH_2CH_{2(B)}CH_3$), 1.03–0.87 (12H, overlap, CH_3-18, CH_3-30, $CH_2CH_2CH_{3(A)}$, $CH_2CH_2CH_{3(B)}$), 0.86 (3H, d, $J = 6.5$ Hz, CH_3-21), 0.72 (1H, d, $J = 4.2$ Hz, H_β-19), 0.37 (1H, d, $J = 4.5$ Hz, H_α-19); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 173.2 (C, C-3), 170.3 (C, C-27), 150.0 (C, C-4), 133.3 (CH, C-24), 132.0 (C, C-25), 111.3 (CH_2, C-28), 52.0 (CH, C-17), 48.9 (C, C-14), 47.5 (CH, C-8), 46.0 (CH, C-5), 45.1 (C, C-13), 41.1 ($C_{(A)}H_2CH_2CH_3$), 41.0 ($C_{(B)}H_2CH_2CH_3$), 36.3 (CH_2, C-11), 35.9 (CH, C-20), 35.5 (CH_2, C-12), 34.0 (CH_2, C-15), 33.0 (CH_2, C-2), 30.0 (CH_2, C-19), 29.7 (CH_2, C-1), 28.0 (CH_2, C-16), 27.7 (CH_2, C-6), 27.1 (C, C-10), 27.0 (CH_2, C-22), 26.3 (CH_2, C-23), 24.9 (CH_2, C-7), 22.9 ($CH_2C_{(A)}H_2CH_3$), 22.8 ($CH_2C_{(B)}H_2CH_3$), 21.3 (C, C-9), 20.9 (CH_3, C-26), 19.7 (CH_3, C-29), 19.2 (CH_3, C-30), 18.1 (CH_3, C-21), 17.9 (CH_3, C-18), 11.4 ($CH_2CH_2C_{(A)}H_3$), 11.3 ($CH_2CH_2C_{(B)}H_3$); HRMS-ESI: *m/z* calcd for $C_{36}H_{60}N_2NaO_2$ $(M+Na)^+$ 575.4547, found 575.4546.

N,N'-dipropyl 3,4-secolanosta-4(28),8,24-(Z)-trien-3,-27-diamide ***(10)***. According to the above general procedures, derivative **10** was prepared via synthesis of MA (**2**) with 1-propanamine; white solid. 31 mg, 56% yield; mp 108–111 °C; IR ν_{max} (KBr) 3441, 2964, 2925, 1641, 1545, 1460, 1403, 1323, 1262, 1101, 1026, 802 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 5.53–5.32 (3H, overlap, $CONH_{(A)}$, $CONH_{(B)}$, H-24), 4.89 (1H, s, H_A-28), 4.68 (1H, s, H_B-28), 3.36–3.24 (2H, m, $J = 7.2$, 2.4 Hz, $CH_{2(A)}CH_2CH_3$), 3.23–3.09 (2H, m, $J = 6.8$ Hz, $CH_{2(B)}CH_2CH_3$), 1.88, 1.76 (6H, s, CH_3-26, CH_3-29), 2.31–1.14 (27H, overlap, manwuweizic acid scaffold, $CH_2CH_{2(A)}CH_3$, $CH_2CH_{2(B)}CH_3$), 0.99–0.86 (15H, overlap, CH_3-18, CH_3-30, CH_3-21, $CH_2CH_2CH_{3(A)}$, $CH_2CH_2CH_{3(B)}$), 0.72 (3H, s, CH_3-19); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 173.4 (C, C-3), 170.3 (C, C-27), 147.6 (C, C-4), 138.8 (C, C-9), 133.4 (CH, C-24), 132.0 (C, C-8), 129.6 (C, C-25), 113.7 (CH_2, C-28), 50.7 (C, C-14), 50.2 (CH, C-17), 47.0 (CH, C-5), 44.3 (C, C-13), 41.2 ($C_{(A)}H_2CH_2CH_3$), 41.0 ($C_{(B)}H_2CH_2CH_3$), 40.4 (C, C-10), 36.3 (CH_2, C-1), 36.3 (CH, C-20), 33.3 (CH_2, C-2), 31.9 (CH_2, C-6), 31.1 (CH_2, C-7), 31.0 (CH_2, C-11), 28.0 (CH_2, C-12), 26.4 (CH_2, C-15), 25.8 (CH_2, C-16), 25.2 (CH_3, C-30), 24.0 (CH_2, C-22), 23.0 (CH_3, C-19), 23.0 ($CH_2C_{(A)}H_2CH_3$), 22.9 ($CH_2C_{(B)}H_2CH_3$), 22.4 (CH_3, C-18), 21.8 (CH_2, C-23), 20.9 (CH_3, C-21), 18.6 (CH_3, C-26), 15.9 (CH_3, C-29), 11.5 ($CH_2CH_2C_{(A)}H_3$), 11.3 ($CH_2CH_2C_{(B)}H_3$); HRMS-ESI: *m/z* calcd for $C_{36}H_{60}N_2NaO_2$ $(M+Na)^+$ 575.4547, found 575.4554.

N,N'-diisobutyl 3,4-secocycloarta-4(28),24-(Z)-diene-3,-27-diamide ***(11)***. According to the above general procedures, derivative **11** was prepared via synthesis of NA (**1**) with isobutylamine; white solid. 41 mg, 71% yield; mp 119–122 °C; IR ν_{max} (KBr) 3425, 2955, 2923, 2867, 1655, 1625, 1548, 1433, 1454, 1385, 1275, 1159, 1072, 1034 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 5.59 (1H, t, $J = 5.2$ Hz, $CONH_{(A)}$), 5.56–5.49 (1H, t, $J = 5.6$ Hz, $CONH_{(B)}$), 5.44 (1H, t, $J = 7.3$ Hz, H-24), 4.80 (1H, s, H_A-28), 4.71 (1H, s, H_B-28), 3.20–3.07 [2H, m, $J = 4.3$, 2.0 Hz, $CH_{2(A)}CH(CH_3)_2$], 3.07–2.94 [2H, m, $J = 6.3$, 1.4 Hz, $CH_{2(B)}CH(CH_3)_2$], 1.87, 1.66 (6H, s, CH_3-26, CH_3-29), 2.47–1.02 [26H, m, nigranoic acid scaffold, $CH_2CH_{(A)}(CH_3)_2$, $CH_2CH_{(B)}(CH_3)_2$], 0.94–0.85 [18H, overlap, CH_3-18, CH_3-30, $CH_2CH(CH_{3(A)})_2$, $CH_2CH(CH_{3(B)})_2$], 0.84 (3H, d, $J = 6.6$ Hz, CH_3-21), 0.70 (1H, d, $J = 4.0$ Hz, H_β-19), 0.37 (1H, d, $J = 4.4$ Hz, H_α-19); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 173.2 (C, C-3), 170.3 (C, C-27), 149.9 (C, C-4), 133.2 (CH, C-24), 132.0 (C, C-25), 111.3 (CH_2, C-28), 52.0 (CH, C-17), 48.9 (C, C-14), 47.4 (CH, C-8), 46.7 [$C_{(A)}H_2CH(CH_3)_2$], 46.6 [$C_{(B)}H_2CH(CH_3)_2$], 45.9 (CH, C-5), 45.1 (C, C-13), 36.2 (CH_2, C-11), 35.9 (CH, C-20), 35.5 (CH_2, C-12), 34.0 (CH_2, C-15), 32.9 (CH_2, C-2), 30.1 (CH_2, C-19), 29.7 (CH_2, C-1), 28.5 [$CH_2C_{(A)}H(CH_3)_2$], 28.4 [$CH_2C_{(A)}H(CH_3)_2$], 28.0 (CH_2, C-16), 27.6 (CH_2, C-6), 27.1 (C, C-10), 26.9 (CH_2, C-22), 26.4 (CH_2, C-23), 24.9 (CH_2, C-7), 21.3 (C, C-9), 20.9 (CH_3, C-26), 20.1 [2C, $CH_2CH(C_{(A)}H_3)_2$], 20.0 [2C, $CH_2CH(C_{(A)}H_3)_2$], 19.6 (CH_3, C-29), 19.2 (CH_3, C-30), 18.1 (CH_3, C-21), 17.9 (CH_3, C-18); HRMS-ESI: *m/z* calcd for $C_{38}H_{64}N_2NaO_2$ $(M+Na)^+$ 603.4860, found 603.4856.

N,N'-diisobutyl 3,4-secolanosta-4(28),8,24-(Z)-trien-3,-27-diamide ***(12)***. According to the above general procedures, derivative **12** was prepared via synthesis of MA (**2**) with isobutylamine; white solid. 33 mg, 57% yield; mp 103–105 °C; IR ν_{max} (KBr) 3442, 2959, 2926, 1639, 1548, 1466, 1372, 1264, 1158, 1031 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 5.54–5.35 (3H, overlap, $CONH_{(A)}$, $CONH_{(B)}$, H-24), 4.90 (1H, s, H_A-28), 4.69 (1H, s, H_B-28), 3.22–3.10 [2H, m, $J = 4.1$, 2.4 Hz, $CH_{2(A)}CH(CH_3)_2$], 3.10–3.01 [2H, m, $J = 6.6$ Hz, $CH_{2(B)}CH(CH_3)_2$], 1.89, 1.76 (6H, s, CH_3-26, CH_3-29), 2.30–1.07 [25H, m, manwuweizic acid scaffold, $CH_2CH_{(A)}(CH_3)_2$, $CH_2CH_{(B)}(CH_3)_2$], 1.01–0.79 [21H, overlap, CH_3-18, CH_3-30, CH_3-21, $CH_2CH(CH_{3(A)})_2$, $CH_2CH(CH_{3(B)})_2$], 0.72 (3H, s, CH_3-19); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 173.4 (C, C-3), 170.3 (C, C-27), 147.7 (C, C-4), 138.8 (C, C-9), 133.4 (CH, C-24), 132.0 (C, C-8), 129.6 (C, C-25), 113.6 (CH_2, C-28), 50.8 (C, C-14), 50.3 (CH, C-17), 47.0 (CH, C-5), 46.8 [$C_{(A)}H_2CH(CH_3)_2$], 46.7 [$C_{(B)}H_2CH(CH_3)_2$], 44.4 (C, C-13), 40.4 (C, C-10), 36.3 (CH, C-20), 36.3 (CH_2, C-21), 33.4 (CH_2, C-2), 32.0 (CH_2, C-6), 31.1 (CH_2, C-7), 31.0 (CH_2, C-11), 28.6 [$CH_2C_{(A)}H(CH_3)_2$], 28.5 [$CH_2C_{(A)}H(CH_3)_2$], 28.0 (CH_2, C-12), 26.5 (CH_2, C-15), 25.9 (CH_2, C-16), 25.2 (CH_3, C-30), 24.0 (CH_2, C-22), 23.0 (CH_3, C-19), 22.4 (CH_3, C-18), 21.8 (CH_2, C-23), 21.0 (CH_3, C-21), 20.2 [2C, $CH_2CH(C_{(A)}H_3)_2$], 20.1 [2C, $CH_2CH(C_{(A)}H_3)_2$], 18.6 (CH_3, C-26), 15.9 (CH_3, C-29);

N,N'-dimethyl 3,4-secocycloarta-4(28),24-(Z)-diene-3,-27-diamide ***(13)***. According to the above general procedures, derivative **13** was prepared via synthesis of NA (**1**) with methylamine hydrochloride; white solid. 38 mg, 76% yield; mp 177–179 °C; IR ν_{max} (KBr) 3356, 2925, 2874, 1653, 1539, 1456, 1407, 1278, 1238, 1155 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 5.61–5.37 (3H, overlap, $CONH_{(A)}$, $CONH_{(B)}$, H-24), 4.80 (1H, s, H_A-28), 4.72 (1H, s, H_B-28), 2.87 (2H, d, $J = 4.8$ Hz, $CONHCH_{3(A)}$), 2.76 (2H, d, $J = 4.5$ Hz, $CONHCH_{3(B)}$), 1.87, 1.67 (6H, s, CH_3-26, CH_3-29), 2.47–1.04 (24H, m, nigranoic acid scaffold), 0.94, 0.91 (6H, s, CH_3-18, CH_3-30), 0.86 (3H, d, $J = 6.4$ Hz, CH_3-21), 0.72 (1H, d, $J = 3.3$ Hz, H_β-19), 0.38 (1H, d, $J = 3.7$ Hz, H_α-19); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 173.9 (C, C-3), 170.9 (C, C-27), 149.9 (C, C-4), 134.0 (CH, C-24), 131.6 (C, C-25), 111.3 (CH_2, C-28), 52.0 (CH, C-17),

49.0 (C, C-14), 47.5 (CH, C-8), 46.0 (CH, C-5), 45.1 (C, C-13), 36.3 (CH_2, C-11), 35.9 (CH, C-20), 35.5 (CH_2, C-12), 33.8 (CH_2, C-15), 33.0 (CH_2, C-2), 29.9 (CH_2, C-19), 29.7 (CH_2, C-1), 28.0 (CH_2, C-16), 27.7 (CH_2, C-6), 27.2 (C, C-10), 27.0 (CH_2, C-22), 26.4 (CH_2, C-23), 26.2 (CONHC$_{(A)}$H$_3$), 26.0 (CONHC$_{(B)}$H$_3$), 24.9 (CH_2, C-7), 21.4 (C, C-9), 20.9 (CH_3, C-26), 19.7 (CH_3, C-29), 19.3 (CH_3, C-30), 18.1 (CH_3, C-21), 17.9 (CH_3, C-18); HRMS-ESI: *m/z* calcd for $C_{32}H_{53}N_2O_2$ $(M+H)^+$ 497.4102, found 497.4107.

*N,N'-dimethyl 3,4-secolanosta-4(28),8,24-(Z)-trien-3,-27-diamide **(14)***. According to the above general procedures, derivative **14** was prepared via synthesis of MA (**2**) with methylamine hydrochloride; white solid. 33 mg, 66% yield; mp 164–167 °C; IR ν_{max} (KBr) 3423, 3320, 2963, 2926, 2881, 1654, 1633, 1560, 1467, 1408, 1323, 1274, 1160, 1026 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 5.60–5.43 (3H, overlap, CONH$_{(A)}$, CONH$_{(B)}$, H-24), 4.88 (1H, s, H$_A$-28), 4.67 (1H, s, H$_B$-28), 2.86 (2H, d, J = 4.4 Hz, CONHCH$_{3(A)}$), 2.78 (2H, d, J = 4.4 Hz, CONHCH$_{3(B)}$), 1.87, 1.75 (6H, s, CH$_3$-26, CH$_3$-29), 2.26–1.03 (23H, m, manwuweizic acid scaffold), 0.94 (3H, s, CH$_3$-18), 0.92–0.84 (6H, overlap, CH$_3$-21, CH$_3$-30), 0.71 (3H, s, CH$_3$-19); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 174.1 (C, C-3), 170.6 (C, C-27), 147.6 (C, C-4), 138.8 (C, C-9), 134.0 (CH, C-24), 131.6 (C, C-8), 129.6 (C, C-25), 113.7 (CH_2, C-28), 50.7 (C, C-14), 50.3 (CH, C-17), 47.0 (CH, C-5), 44.4 (C, C-13), 40.4 (C, C-10), 36.3 (CH_2, C-1), 36.2 (CH, C-20), 33.2 (CH_2, C-2), 31.7 (CH_2, C-6), 31.1 (CH_2, C-7), 31.0 (CH_2, C-11), 28.0 (CH_2, C-12), 26.4 (CH_2, C-15), 26.3 (CONHC$_{(A)}$H$_3$), 26.1 (CONHC$_{(B)}$H$_3$), 25.8 (CH_2, C-16), 25.2 (CH_3, C-30), 24.0 (CH_2, C-22), 23.0 (CH_3, C-19), 22.3 (CH_3, C-18), 21.8 (CH_2, C-23), 20.9 (CH_3, C-21), 18.6 (CH_3, C-26), 15.9 (CH_3, C-29); HRMS-ESI: *m/z* calcd for $C_{32}H_{53}N_2O_2$ $(M+H)^+$ 497.4102, found 497.4100.

*N,N'-diethyl 3,4-secocycloarta-4(28),24-(Z)-diene-3,-27-diamide **(15)***. According to the above general procedures, derivative **15** was prepared via synthesis of NA (**1**) with ethylamine hydrochloride; white solid. 28 mg, 53% yield; mp 140–141 °C; IR ν_{max} (KBr) 3306, 3073, 2924, 2873, 1650, 1535, 1454, 1374, 1323, 1264, 1239, 1148, 890 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 5.54–5.42 (2H, overlap, CONH$_{(A)}$, H-24), 5.40–5.32 (1H, m, CONH$_{(B)}$), 4.81 (1H, s, H$_A$-28), 4.73 (1H, s, H$_B$-28), 3.42–3.30 (2H, m, J = 6.6, 1,3 Hz, CH$_{2(A)}$CH$_3$), 3.29–3.20 (2H, m, J = 6.9, 1,5 Hz, CH$_{2(B)}$CH$_3$), 1.88, 1.68 (6H, s, CH$_3$-26, CH$_3$-29), 2.47–1.22 (24H, m, nigranoic acid scaffold), 1.18 (3H, t, J = 7.3 Hz, CH$_2$CH$_{3(A)}$), 1.11 (3H, t, J = 7.2 Hz, CH$_2$CH$_{3(B)}$), 0.94, 0.91 (6H, s, CH$_3$-18, CH$_3$-30), 0.87 (3H, d, J = 6.3 Hz, CH$_3$-21), 0.72 (1H, d, J = 4.2 Hz, H$_\beta$-19), 0.39 (1H, d, J = 4.4 Hz, H$_\alpha$-19); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 173.1 (C, C-3), 170.2 (C, C-27), 150.0 (C, C-4), 133.5 (CH, C-24), 131.9 (C, C-25), 111.4 (CH_2, C-28), 52.0 (CH, C-17), 49.0 (C, C-14), 47.5 (CH, C-8), 46.0 (CH, C-5), 45.1 (C, C-13), 36.3 (CH_2, C-11), 35.9 (CH, C-20), 35.5 (CH_2, C-12), 34.3 (C$_{(A)}$H$_2$CH$_3$), 34.2 (C$_{(B)}$H$_2$CH$_3$), 34.0 (CH_2, C-15), 33.0 (CH_2, C-2), 30.0 (CH_2, C-19), 29.7 (CH_2, C-1), 28.1 (CH_2, C-16), 27.7 (CH_2, C-6), 27.2 (C, C-10), 27.0 (CH_2, C-22), 26.3 (CH_2, C-23), 24.9 (CH_2, C-7), 21.4 (C, C-9), 20.9 (CH_3, C-26), 19.7 (CH_3, C-29), 19.3 (CH_3, C-30), 18.2 (CH_3, C-21), 17.9 (CH_3, C-18), 15.0 ($CH_2C_{(A)}H_3$), 14.9 ($CH_2C_{(B)}H_3$); HRMS-ESI: *m/z* calcd for $C_{34}H_{57}N_2O_2$ $(M+H)^+$ 525.4415, found 525.4412.

*N,N'-diethyl 3,4-secolanosta-4(28),8,24-(Z)-trien-3,-27-diamide **(16)***. According to the above general procedures, derivative **16** was prepared via synthesis of MA (**2**) with ethylamine hydrochloride; white solid. 33 mg, 63% yield; mp 136–139 °C; IR ν_{max} (KBr) 3425, 2963, 2924, 2853, 1642, 1402, 1323, 1262, 1097, 1030, 802 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 5.56–5.37 (3H, overlap, CONH$_{(A)}$, CONH$_{(B)}$, H-24), 4.88 (1H, s, H$_A$-28), 4.68 (1H, s, H$_B$-28), 3.40–3.29 (2H, m, J = 7.1 Hz, CH$_{2(A)}$CH$_3$), 3.29–3.20 (2H, m, J = 7.2 Hz, CH$_{2(B)}$CH$_3$), 1.87, 1.75 (6H, s, CH$_3$-26, CH$_3$-29), 2.30–1.19 (23H, m, manwuweizic acid scaffold), 1.17 (3H, t, J = 7.1 Hz, CH$_2$CH$_{3(A)}$), 1.11 (3H, t, J = 7.2 Hz, CH$_2$CH$_{3(B)}$), 0.94, 0.71 (6H, s, CH$_3$-18, CH$_3$-19), 0.92–0.84 (6H, overlap, CH$_3$-21, CH$_3$-30); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 173.3 (C, C-3), 170.1 (C, C-27), 147.6 (C, C-4), 138.7 (C, C-9), 133.4 (CH, C-24), 131.9 (C, C-8), 129.7 (C, C-25), 113.6 (CH_2, C-28), 50.7 (C, C-14), 50.3 (CH, C-17), 47.0 (CH, C-5), 44.4 (C, C-13), 40.4 (C, C-10), 36.3 (CH_2, C-1), 36.3 (CH, C-20), 34.3 (C$_{(A)}$H$_2$CH$_3$), 34.1 (C$_{(B)}$H$_2$CH$_3$), 33.3 (CH_2, C-2), 31.8 (CH_2, C-6), 31.1 (CH_2, C-7), 31.0 (CH_2, C-11), 28.0 (CH_2, C-12), 26.3 (CH_2, C-15), 25.8 (CH_2, C-16), 25.2 (CH_3, C-30), 24.0 (CH_2, C-22), 23.0 (CH_3, C-19), 22.3 (CH_3, C-18), 21.8 (CH_2, C-23), 20.8 (CH_3, C-21), 18.6 (CH_3, C-26), 15.9 (CH_3, C-29), 14.9 ($CH_2C_{(A)}H_3$), 14.9 ($CH_2C_{(B)}H_3$); HRMS-ESI: *m/z* calcd for $C_{34}H_{57}N_2O_2$ $(M+H)^+$ 525.4415, found 525.4417.

*N,N'-diallyloxy 3,4-secocycloarta-4(28),24-(Z)-diene-3,-27-diamide **(17)***. According to the above general procedures, derivative **17** was prepared via synthesis of NA (**1**) with *O*-allylhydroxylamine hydrochloride; white solid. 30 mg, 52% yield; mp 110–112 °C; IR ν_{max} (KBr) 3442, 2925, 1640, 1453, 1262, 1031, 929 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 8.16 (2H, s, CONH$_{(A)}$ and CONH$_{(B)}$), 6.06–5.88 (2H, overlap, OCH$_2$CH$_{(A)}$═CH$_2$ and OCH$_2$CH$_{(B)}$═CH$_2$), 5.57 (1H, t, J = 7.5 Hz, H-24), 5.41–5.26 (4H, overlap, OCH$_2$CH═CH$_{2(A)}$ and OCH$_2$CH═CH$_{2(B)}$), 4.82 (1H, s, H$_A$-28), 4.74 (1H, s, H$_B$-28), 4.51–4.41 (2H, m, OCH$_{2(A)}$—CH═CH$_2$), 4.41–4.28 (2H, m, OCH$_{2(B)}$—CH═CH$_2$), 1.88, 1.68 (6H, s, CH$_3$-26, CH$_3$-29), 2.50–1.05 (24H, m, nigranoic acid scaffold), 0.95, 0.92 (6H, s, CH$_3$-18, CH$_3$-30), 0.88 (3H, d, J = 6.0 Hz, CH$_3$-21), 0.73 (1H, d, J = 3.2 Hz, H$_\beta$-19), 0.41 (1H, d, J = 3.2 Hz, H$_\alpha$-19); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 171.3 (C, C-3), 168.2 (C, C-27), 149.8 (C, C-4), 136.0 (CH, C-24), 132.2 (OCH$_2$C$_{(A)}$H═CH$_2$), 129.9 (OCH$_2$C$_{(B)}$H═CH$_2$), 128.5 (C, C-25), 120.8 (OCH$_2$CH═C$_{(A)}$H$_2$), 120.7 (OCH$_2$CH═C$_{(B)}$H$_2$), 111.5 (CH_2, C-28), 77.5 (overlap, OC$_{(A)}$H$_2$CH═CH$_2$), 77.2 (overlap, OC$_{(B)}$H$_2$CH═CH$_2$), 52.1 (CH, C-17), 49.0 (C, C-14), 47.5 (CH, C-8), 46.1 (CH, C-5), 45.2 (C, C-13), 36.2 (CH_2, C-11), 35.9 (CH, C-20), 35.5 (CH_2, C-12), 33.0 (CH_2, C-15), 33.0 (CH_2, C-2), 29.8 (CH_2, C-19), 29.3 (CH_2, C-1), 28.1 (CH_2, C-16), 27.7 (CH_2, C-6), 27.2 (C, C-10), 27.0 (CH_2, C-22), 26.6 (CH_2, C-23), 24.9 (CH_2, C-7), 21.4 (C, C-9), 20.6 (CH_3, C-26), 19.7 (CH_3, C-29), 19.3 (CH_3, C-30), 18.2 (CH_3, C-21), 18.0 (CH_3, C-18); HRMS-ESI: *m/z* calcd for $C_{36}H_{56}N_2NaO_4$ $(M+Na)^+$ 603.4132, found 603.4129.

*N,N'-diallyloxy 3,4-secocycloarta-4(28),8,24-(Z)-diene-3,-27-diamide **(18)***. According to the above general procedures, derivative **18** was prepared via synthesis of MA (**2**) with *O*-allylhydroxylamine hydrochloride; white solid. 32 mg, 55% yield; mp 103–105 °C; IR ν_{max} (KBr) 3440, 2923, 1640, 1402, 1324, 1029, 929 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 8.06 (2H, s, CONH$_{(A)}$ and CONH$_{(B)}$), 6.12–5.81 (2H, overlap, OCH$_2$CH$_{(A)}$═CH$_2$ and OCH$_2$CH$_{(B)}$═CH$_2$), 5.57 (1H, t, J = 7.3 Hz, H-24), 5.43–5.22 (4H, overlap, OCH$_2$CH═CH$_{2(A)}$ and OCH$_2$CH═CH$_{2(B)}$), 4.89 (1H, s, H$_A$-28), 4.67 (1H, s, H$_B$-28), 4.51–4.41 (2H, m, OCH$_{2(A)}$—CH═CH$_2$), 4.41–4.26 (2H, m, OCH$_{2(B)}$—CH═CH$_2$), 1.87, 1.75 (6H, s, CH$_3$-26, CH$_3$-29), 2.31–1.04 (23H, m, manwuweizic acid scaffold), 0.95, 0.72 (6H, s, CH$_3$-18, CH$_3$-19), 0.93–0.84 (6H, overlap, CH$_3$-21, CH$_3$-30); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 171.6 (C, C-3), 168.2 (C, C-27), 147.5 (C, C-4), 139.1 (C, C-9), 136.0 (CH, C-24), 132.2 (OCH$_2$-C$_{(A)}$H═CH$_2$), 129.9 (OCH$_2$C$_{(B)}$H═CH$_2$), 129.5 (C, C-8), 128.5 (C, C-25), 120.8 (OCH$_2$CH═C$_{(A)}$H$_2$), 120.8 (OCH$_2$CH═C$_{(B)}$H$_2$), 113.8 (CH_2, C-28), 77.5 (overlap, OC$_{(A)}$H$_2$CH═CH$_2$), 77.2 (overlap, OC$_{(B)}$H$_2$CH═CH$_2$), 50.8 (C, C-14), 50.3 (CH, C-17), 47.1 (CH, C-5), 44.4 (C, C-13), 40.5 (C, C-10), 36.3 (CH_2, C-1), 36.2 (CH, C-20), 29.8 (CH_2, C-2), 29.3 (CH_2, C-6), 31.1 (CH_2, C-7), 31.0 (CH_2, C-11), 28.1 (CH_2, C-12), 26.6 (CH_2, C-15), 25.9 (CH_2, C-16), 25.3 (CH_3, C-30), 24.0 (CH_2, C-22), 23.0 (CH_3, C-19), 22.3 (CH_3, C-18), 21.8 (CH_2, C-23), 20.6 (CH_3, C-21), 18.6 (CH_3, C-26), 16.0 (CH_3, C-29); HRMS-ESI: *m/z* calcd for $C_{36}H_{56}N_2NaO_4$ $(M+Na)^+$ 603.4132, found 603.4128.

*4.1.4. Procedures for the synthesis of **19** and **20***

To a solution of diesters (**1a**, 88 mg, 0.18 mmol) and hydroxylamine solution (50 wt. % in Water, 0.46 mL, 7.2 mmol) in a mixed solvent (THF-methanol, 1:1, 2 mL) was added KOH (81 mg, 1.44 mmol). The mixture was stirred at room temperature for 3 h, acidified to pH 7 with 1 N HCl solution and extracted with EtOAc (3 × 10 mL). The combined organic layers were washed with brine (20 mL). The organic phase was dried over anhydrous Na_2SO_4. Filtration and evaporation of the solvent at reduced pressure gave a crude product, which was purified by column chromatography (DCM-methanol, 15:1) to give the pure compound **19**. Compound **20** was prepared from **2a (**88 mg, 0.18 mmol) with the same

procedure as that for **19**.

*3,4-secocycloarta-4(28),24-(Z)-diene-3-(hydroxyamino)-27-oic acid **(19)***. white solid. 33 mg, 38% yield; mp 116–121 °C; IR ν_{max} (KBr) 3440, 3217, 2931, 1687, 1633, 1542, 1457, 1375, 1259, 1073, 993, 891 cm^{-1}; ^1H NMR (Pyridine-d_5, 400 MHz) δ 6.07 (1H, t, J = 6.9 Hz, H-24), 4.93 (1H, s, H_A-28), 4.79 (1H, s, H_B-28), 2.17, 1.70 (6H, s, CH_3-26, CH_3-29), 2.97–1.14 (24H, m, nigranoic acid scaffold), 0.99 (3H, d, J = 6.3 Hz, CH_3-21), 0.95, 0.85 (6H, s, CH_3-18, CH_3-30), 0.67 (1H, d, J = 4.0 Hz, H_β-19), 0.37 (1H, d, J = 4.0 Hz, H_α-19); ^{13}C NMR (Pyridine-d_5, 100 MHz) δ 171.2 (C, C-3), 171.2 (C, C-27), 150.6 (C, overlap, C-4), 143.2 (CH, C-24), 129.2 (C, C-25), 112.4 (CH_2, C-28), 52.9 (CH, C-17), 49.6 (C, C-14), 48.2 (CH, C-8), 46.4 (CH, C-5), 45.8 (C, C-13), 36.9 (CH_2, C-11), 36.9 (CH, C-20), 36.3 (CH_2, C-12), 33.7 (CH_2, C-15), 31.7 (CH_2, C-2), 31.1 (CH_2, C-19), 30.5 (CH_2, C-1), 28.8 (CH_2, C-16), 28.4 (CH_2, C-6), 28.3 (C, C-10), 27.7 (CH_2, C-22), 27.5 (CH_2, C-23), 25.7 (CH_2, C-7), 22.0 (C, C-9), 22.0 (CH_3, C-26), 20.5 (CH_3, C-29), 19.9 (CH_3, C-30), 18.9 (CH_3, C-21), 18.7 (CH_3, C-18); HRMS-ESI: *m/z* calcd for $C_{30}H_{47}NNaO_4$ $(M+Na)^+$ 508.3397, found 508.3397.

*3,4-secolanosta-4(28),8,24-(Z)-trien-3-(hydroxyamino)-27-oic acid **(20)***. white solid. 25 mg, 30% yield; mp 114 °C; IR ν_{max} (KBr) 3443, 2925, 1640, 1401, 1323, 1265 cm^{-1}; ^1H NMR (Pyridine-d_5, 400 MHz) δ 6.07 (1H, t, J = 6.9 Hz, H-24), 4.96 (1H, s, H_A-28), 4.88 (1H, s, H_B-28), 2.16, 1.79 (6H, s, CH_3-26, CH_3-29), 3.05–1.12 (23H, m, manwuweizic acid scaffold), 1.03 (3H, d, J = 5.2 Hz, CH_3-21), 0.98, 0.86, 0.76 (9H, s, CH_3-18, CH_3-30, CH_3-19); ^{13}C NMR (Pyridine-d_5, 100 MHz) δ 171.5 (C, C-3), 171.2 (C, C-27), 148.3 (C, C-4), 143.2 (CH, C-24), 139.2 (C, C-9), 130.7 (C, C-8), 129.2 (C, C-25), 114.7 (CH_2, C-28), 51.5 (C, C-14), 51.1 (CH, C-17), 47.4 (CH, C-5), 45.0 (C, C-13), 41.2 (C, C-10), 37.2 (CH, C-20), 36.9 (CH_2, C-1), 34.7 (CH_2, C-2), 31.8 (CH_2, C-6), 31.8 (CH_2, C-7), 29.5 (CH_2, C-11), 28.8 (CH_2, C-12), 27.5 (CH_2, C-15), 26.6 (CH_2, C-16), 25.8 (CH_3, C-30), 24.8 (CH_2, C-22), 23.9 (CH_3, C-19), 23.0 (CH_3, C-18), 22.5 (CH_2, C-23), 22.0 (CH_3, C-21), 19.4 (CH_3, C-26), 16.7 (CH_3, C-29); HRMS-ESI: *m/z* calcd for $C_{30}H_{48}NO_4$ $(M+H)^+$ 486.3578, found 486.3578.

4.2. Virtual screening

To identify HDAC inhibitors from an in-house natural products library containing 1086 molecules, a structure-based virtual screening was performed using FlexX molecular docking program [22]. The receptor model was built based on the crystal structure of HDAC2 (PDB ID: 4LXZ) [23], with the pocket composed of residues within 6.5 Å around the reference ligand (SAHA) cocrystalized in 4LXZ. The coordination of Zn^{2+} was set to spherical. Pharmacophore constraints were defined to include the interaction between the ligand and Zn^{2+}, His145 and His146. The conformation optimization for ligands was performed with Discovery Studio (Accelrys, San Diego, CA, USA). Docking parameters remained as default. Top 100 compounds with lowest binding energy were clustered to 30 clusters by Discovery Studio, and 0–2 compounds in each cluster were selected by considering structure diversity and compound amount. Finally, 10 compounds were chosen for experimental validation.

4.3. Molecular docking for binding mode analysis

To understand the interaction of active compounds with different HDAC subtypes, the same molecular docking protocol was applied as for virtual screening. The receptor models were built with following structures (PDB ID): 5ICN [24] for HDAC1, 4LXZ [23] for HDAC2, 2VQM [25] for HDAC4, 5EDU [26] for HDAC6.

4.4. Biological study

4.4.1. HDAC inhibition assay

The in vitro HDAC inhibitory activity was tested by ChemPartner Co., Ltd, shanghai, People's Republic of China. SAHA was used as the reference compound for HDAC1, HDAC2, HDAC6 and HDAC8. TMP269 was used as the reference compound for HDAC4. For preliminary screening, the inhibition rates at single compound concentration (100 μM) were tested in duplicates. For IC_{50} estimations, six concentrations were measured for each compound, with the starting point of 50 μM and gradient five-fold dilution. The substrate solution was made by adding trypsin and Ac-peptide substrate in Tris buffer. To perform the reaction, firstly 15 μL of enzyme solution was transferred to assay plate with compound solution, and incubated at room temperature for 15 min. Then 10 μL of substrate solution was added to each well and incubated at room temperature for 60 min. The plate was read by Synergy MX with excitation at 355 nm and emission at 460 nm. The inhibition values were obtained by using Equation (1): Inh%=(Max-Signal)/ (Max-Min)*100. The IC_{50} values were obtained by fitting the data in GraphPad using Equation (2): Y = Bottom + (Top-Bottom) / (1 + 10^ ((LogIC$_{50}$-X)*Hill Slope)), where Y is %inhibition and X is compound concentration.

4.4.2. NLRP3 inflammasome activation

Mouse mononuclear macrophages J774A.1 cells were placed in a six-well plate at a density of 1×10^6/ml and cultured overnight. Then, discarded all the supernatant in the six-well plate. The remaining part was pre-stimulated with the opti-MEM (1 mL/well) which contains LPS (200 ng/ml) for 3 h. After the stimulation was complete, discarded again all the supernatant. Added the remaining part into the opti-MEM (500 μL/well) containing either the synthesized compounds which need to be tested or the positive control compound SAHA and pre-incubated for 0.5 h. Then Nig (500 μL per well), the NLRP3 inflammasome stimulator, was added at the final concentration of 10 μM, and incubated with the compound for 1 h [27]. After that, collected all the supernatant, a part of it was used to directly measure the release of LDH (Promega, USA) and IL-1β (mouse IL-1β/IL-1F2 DuoSet ELISA kit R&D Incorporation, USA) [28]. The remaining supernatant was carried out by TCA for protein concentration. The levels of IL-1β and Caspase-1 were detected by Western Blot. Total histone was extracted by EpiQuik Total Histone Extraction Kit. The histone acetylation level was analyzed by Western Blot. The total protein lysate was used as an internal reference by Coomassie blue staining.

4.4.3. Cytotoxicity assay

Cell viability studies induced by synthesized compounds were evaluated by MTT assay. J774A.1 macrophages were seeded in 96-well plated at a density of 4×10^4 cells/well in complete medium and incubated overnight. Each group consisted of 3 wells. Then the cells were treated with synthesized compounds (2.5, 5, 10, 20 or 40 μM) for 48 h. MTT (5 mg/mL in PBS, 10% total volume) was added to each well and the cells were further incubated for 4 h. The supernatant was removed and the cells were lysed with 150 μL/well DMSO. The optical density was measured at 540 (Measurement wavelength) and 630 (reference wavelength) nm on a microplate reader (Thermo Scientifc, Waltham, MA, USA). The IC50 values were calculated using GraphPad Prism 7.

4.4.4. Lactate dehydrogenase (LDH) assay

After inflammasome activation with the treatment of compounds, cell culture supernatants were collected and analyzed by a LDH assay kit (Promega) according the manufacturer's instructions.

4.4.5. Statistical analysis

All values were expressed in the form of mean ± standard deviation, and the analysis software was GraphPad Prism 7.0. One-way analysis of variance (ANOVA) followed by Tukey post hoc test was used to analyze the statistical significance among multiple groups. P-values < 0.05 were considered to indicate statistical significance.

4.4.6. ELISA xxx

The supernatant of the cell culture was taken out and stored in the

refrigerator at −70 °C. IL-1β ELISA kit (R&D DouSet Mouse IL-1β inhibitor/IL-1F2) was used to detect the concentration of IL-1 in the supernatant according to the instructions of the kit.

4.4.7. Biological experiment material

J774A.1 cells were purchased from Kunming Institute of Zoology. SAHA and MCC950 purchased from sigma.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioorg.2021.104728.

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