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Design and SAR of withangulatin A analogs that act as covalent TrxR inhibitors through Michael addition reaction showing potential in cancer treatment

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ABSTRACT

The thioredoxin system plays an important role in cancer cells. Inhibiting thioredoxin reductase (TrxR) has emerged as an effective strategy to selectively target cancer cells. Withangulatin A (**WA**), a natural product extracted from the whole herb of *Physalis angulata* L. (Solanaceae) exhibits potent anticancer activity and other diverse pharmacological activities. To improve activity and targeting, we designed and prepared 41 semisynthetic analogs of WA. Biological evaluation indicated that the most promising compound **13a** displayed the most significant effect on HT-29 cells (human colon cancer cells) ($IC_{50} = 0.08\ \mu M$). A structure-activity relationship study indicated that α, β-unsaturated ketones and ester are necessary groups, allowing **13a** to undergo Michael addition reactions with mercaptan and selenol. LC-MS analysis confirmed that **13a** modified selenocysteine 498 (U) residues in the redox centers of TrxR, resulting in enzyme inhibition. Therefore, compound **13a** acts a novel TrxR inhibitor and may be a promising candidate for cancer intervention.

INTRODUCTION

In the body and cells of humans, reactive oxygen species (ROS) are natural byproducts of oxidative respiration and can function in signal transduction and pathogen clearance during innate immune responses. Compared with noncancerous cells, cancer cells have been reported to harbor increased levels of ROS.[1] ROS usually maintain a high but appropriate level in various cancer cells, primarily due to their active metabolism in response to oncogenic signals.[2] In fact, cancer cells take advantage of this appropriate level of oxidative stress for several significant biological activities, such as proliferation, angiogenesis, and metastasis.[3] However, sustained high levels of ROS can irreversibly damage DNA and lipids and ultimately cause cancer cell apoptosis.[4] The alteration of the redox environment has been suggested to be the 'Achilles' heel' of cancer cells and could be exploited to target them for cancer therapy. Thus, there is increasing interest in modulating cellular redox signaling as a potential strategy for various cancer treatments.[5]

The major source of ROS is mitochondrial metabolism within the cells. The cascade of reversible redox reactions of ROS with the thiol in thiocysteine (Cys) side chains, the selenol in selenocysteine (Sec) side chains, or the sulfide in methionine (Met) side chains make ROS important messenger molecules at the physiological level that regulate diverse redox signaling pathways involved in biological processes.[5] The thioredoxin (Trx) system, one of the key antioxidant systems, is composed of thioredoxin reductase (TrxR), Trx, and NADPH and regulates numerous cellular signaling pathways involved in cell proliferation and survival.[6-8] Mammalian TrxRs are unique large selenoproteins (MW, 114000 Dalton or larger) containing a unique catalytically active selenolthiol/selenenylsulfide in the conserved C-terminal sequence -Gly-Cys-Sec-Gly.[9,10] Trxs from mammalian cells are proteins with a MW of 12000 Dalton that contain a conserved pair of cysteine residues (-CGPC-) in the active site and act as the major disulfide

reductase responsible for maintaining cytosolic proteins in their reduced state.[11] TrxR is the only known physiological enzyme that catalyzes the reduction of oxidized Trx. The Trx system is often overexpressed in most tumors.[12,13] The level of TrxR in cancer cells is often increased by 10-fold or even more compared with that in normal tissues, and cancer proliferation seems to be crucially dependent on an active Trx system, making it a potential target for anticancer drug discovery.[14]

Natural products and their derivatives are an invaluable source of therapeutic agents and have driven pharmaceutical discovery over the past century. The diversity of natural products continues to provide a unique source of bioactive lead compounds for drug development.[15,16] Many compounds used in cancer chemotherapy are derived from natural products, such as paclitaxel, camptothecin, and etoposide. *Physalis angulata L*. is a branched annual shrub that belongs to the Solanaceae family and is distributed worldwide throughout tropical and subtropical regions.[17] It is a common traditional Chinese medicine and has been used for the treatment of conditions such as tumors, hepatitis, rheumatism, nephronia and ulcers.[18] Withangulatin A (Figure 1, **WA, 1**), a group of C28 steroids, is a natural withanolide isolated from *P. angulata* L.[19] WA can exert anticancer effect by changing cell morphology[20] and inhibiting topoisomerase.[17] Its analogues physapubescin B have been reported to induce cell cycle G2/M arrest in combination with GSH.[21] Previous studies have shown that WA analogues possesses potent anticancer activity by consuming GSH and generating ROS via the α, β-unsaturated ketones in ring A.[21,22] The evidence suggests that WA can combine mercaptan and selenol through Michael addition reaction. Despite the well-documented anticancer activity of WA, its target specificity and efficacy are moderate. Hence, it is necessary to improve the potency and increase the targeting of **WA** for future development. In this study, we report the preparation and biological evaluation of 41 semisynthetic analogs of this fascinating class of cytotoxic agents. The cytotoxicity of many derivatives was higher than that of the parent,

and some of the compounds showed good TrxR inhibition. Compound **13a** acts as a novel TrxR inhibitor and may be a promising candidate for cancer intervention.

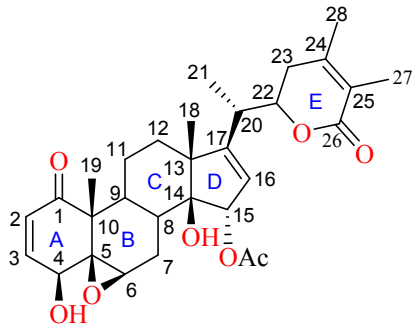


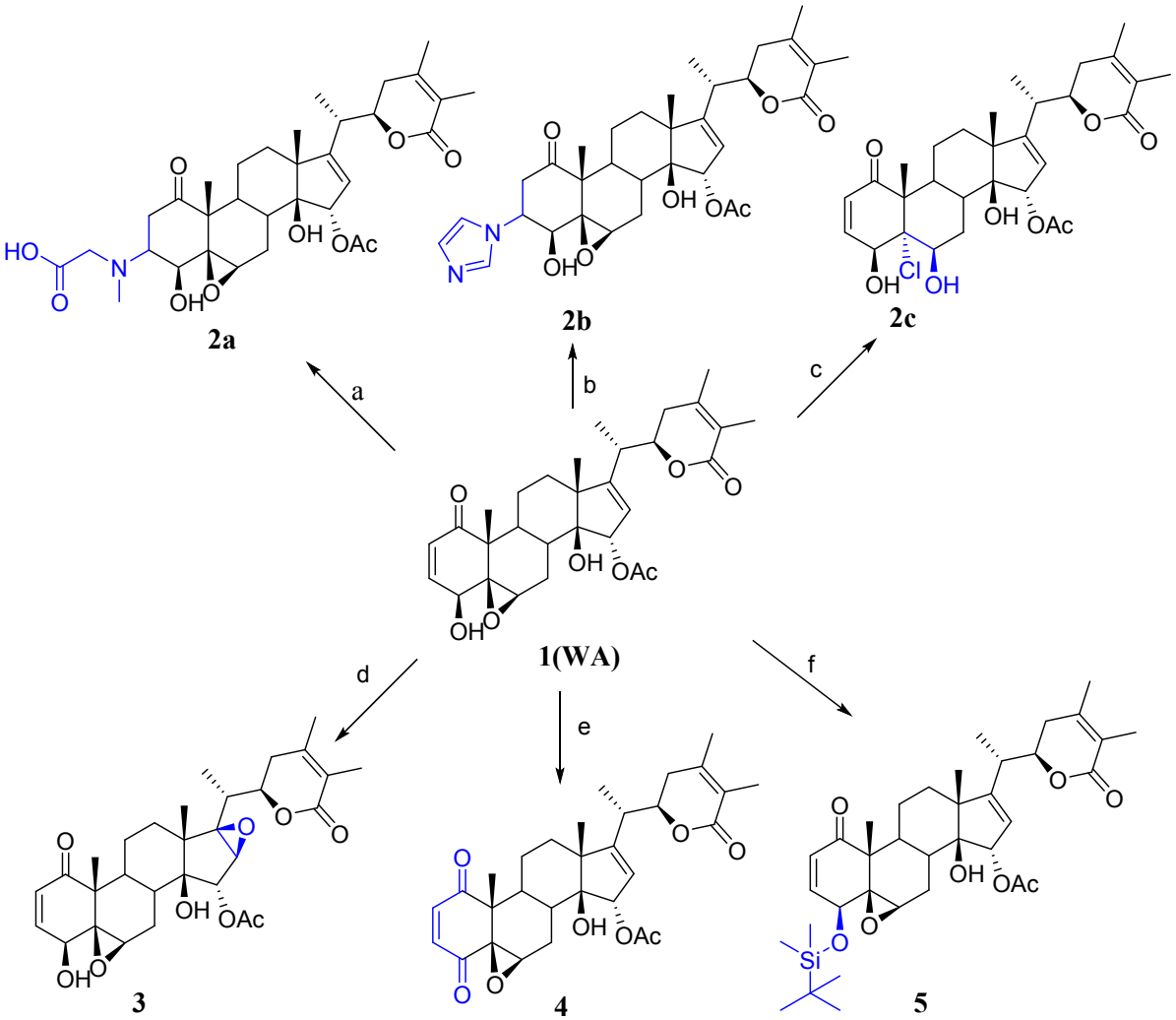
Figure 1. The natural product Withangulatin A (**WA, 1**).

RESULTS AND DISCUSSION

The initial modification and biological evaluation of WA analogs 2-5

Studies have asserted that a 2-ene-1-one and a 5β, 6β-epoxide are essential for the anticancer activity of withanolides.[23-25] Structure-activity relationship (SAR) analysis of the activity of withanolide E against renal carcinoma has been performed.[26] 2-Ene-1-one easily reacts with mercaptoyl through Michael addition, and it is an essential group to induce cells to produce ROS. We initiated (Scheme 1) our study by destroying the α, β-unsaturated ketone system (**2a**, **2b**) and the epoxide (**2c**). The biological results (Table 1) asserted that the α, β-unsaturated ketones and the epoxide are the essential pharmacophores that should not be changed. When we kept the groups and modified the double bond C16=C17 to an epoxide (**3**, the crystal structure and the related crystal data of **3** is shown in Figure S1 and Table S1, respectively) or changed the hydroxyl group to a carbonyl group (**4**), the cytotoxic activity was not improved. A slight change in the biological activity was observed when the only modification was on the hydroxy group (**5**). This finding prompted us to perform the next modification focused on adding different substituents to the hydroxy position.

Scheme 1. Preliminary Synthesis of WA Analogs 2 - 5. [a]



[a] Reagents and conditions: (a) $NH(CH_3)CH_2COOH$, anhydrous CH_2Cl_2, rt, 2h; (b) imidazole, anhydrous CH_2Cl_2, rt, 2h; (c) HCl solution in ether, anhydrous CH_2Cl_2, rt, 1h; (d) mCPBA anhydrous CH_2Cl_2, 0 ºC, 12h; (e) MnO_2, EtOAc, $CHCl_3$, rt, 8h; (f) TBDMSCl, imidazole, anhydrous CH_2Cl_2, rt, 12h.

Table 1. Cytotoxic Activity (IC_{50} Values in μM) of 1-5 against Four Cell Lines. [a]

Compd	Yield (%)	HT-29	HCT-116	HepG2	MCF-7
1(WA)	NP [b]	2.48 ± 0.23	2.37 ± 0.22	2.85 ± 0.12	6.32 ± 0.74
2a	85	> 20.00	> 20.00	> 20.00	> 20.00
2b	85	> 20.00	> 20.00	> 20.00	> 20.00
2c	60	> 20.00	> 20.00	> 20.00	> 20.00
3	78	4.55 ± 0.66	5.04 ± 1.02	4.66 ± 0.45	9.36 ± 1.09
4	81	3.50 ± 0.08	3.11 ± 0.10	6.05 ± 0.10	> 20.00
5	41	1.06 ± 0.04	1.77 ± 0.08	3.01 ± 0.21	6.14 ± 0.78

[a] Cell viability was determined by the MTT assay after 24 h of treatment.

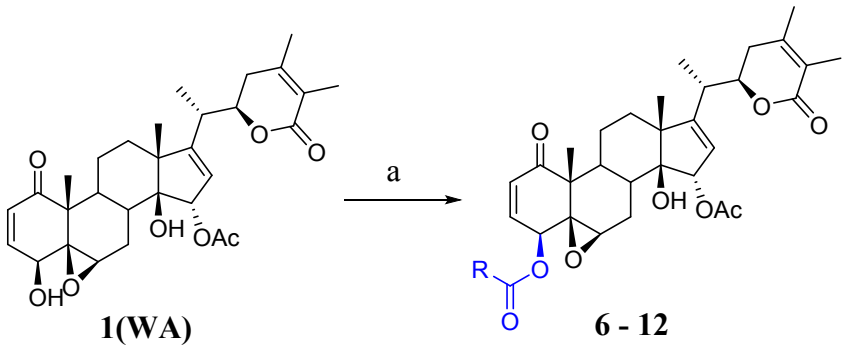
[b] Natural product, 58 mg from 1.0 kg of the dried whole *P. angulata* L. var. *villosa* plant.

Esterification of WA and biological evaluation of analogs 6-12

The next step focused on modifications at position 4-OH. Compounds **6-12** were synthesized as outlined in Scheme 2. These compounds are all the esterification products of WA. After screening the cytotoxic activity against four cancer cell lines (Table 2), most compounds were found to be more active than their parent, and three compounds, **8a**, **9a** and **10a** attracted our attention. **8a** having an α, β-unsaturated carbonyl group, **9a** with a dimethylcarbamate group, and **10a** having a boc amine moiety were the most potential compounds in this series (Table 2). These results suggest that different substituents at position 4 significantly affected the activity of the compound in cancer cells. The analysis of specific structural segments revealed that the introduction of an α, β-unsaturated ketone at 4-OH (**8a**) greatly improved the bioactivity of the compound against the four cell lines. In addition to other factors, this result suggested that the Michael acceptor, an α, β-unsaturated ketone, in the chain more favorably formed covalent interactions than did the ketone in the skeleton of WA, presenting an opportunity for us to introduce new types of modifications. On the other hand, we noticed that the introduction of carbamates (**9a**) or boc amino acid fragment

(**10a**) usually increased the activity of the compounds to some degree, which is another opportunity to optimize our compounds.

Scheme 2. Synthesis of WA Analogs 6-12. [a]



(a) For **6**: CDI, CH_3OH, anhydrous CH_2Cl_2, rt, 8h; For **7a**: $(CH_3CO)_2O$, Et_3N, anhydrous CH_2Cl_2, rt, 12h; For **7b**: $(CH_3CH_2CO)_2O$, Et_3N, anhydrous CH_2Cl_2, rt, 16h; For **8a**, **10**, **11** and **12**: R-COOH, DMAP, EDCI, Et_3N, anhydrous CH_2Cl_2, 0 ºC, 25h; For **9**: $N(CH_3)_2COCl$, Et_3N, anhydrous CH_2Cl_2, 0 ºC, 12h.

Table 2. Cytotoxic Activity (IC_{50} Values in μM) of 6-12 against Four Cell Lines. [a]

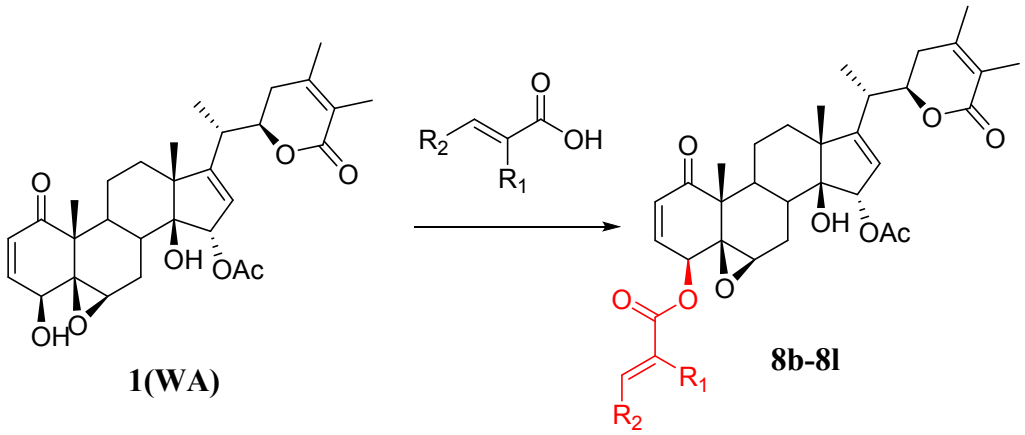
Compd	R	Yield (%)	HT-29	HCT-116	HepG2	MCF-7
6	OCH_3	72	1.79 ± 0.04	1.95 ± 0.07	2.81 ± 0.11	4.33 ± 0.08
7a	CH_3	85	0.80 ± 0.07	0.60 ± 0.09	1.57 ± 0.15	3.05 ± 0.24
7b	CH_2CH_3	76	1.08 ± 0.12	0.57 ± 0.11	1.66 ± 0.03	4.81 ± 0.11
8a	$CH{=}CH_2$	71	0.78 ± 0.15	0.33 ± 0.07	0.52 ± 0.09	1.06 ± 0.12
9a	$N(CH_3)_2$	75	1.01 ± 0.08	0.67 ± 0.06	1.06 ± 0.14	2.10 ± 0.13
10a	CHNHBoc	64	0.63 ± 0.04	0.47 ± 0.06	0.64 ± 0.09	2.16 ± 0.34
11		37	2.39 ± 0.11	3.87 ± 0.45	2.91 ± 0.12	4.08 ± 0.19
12	CH_2CH_2Ph	32	4.05 ± 0.33	5.84 ± 0.41	5.76 ± 0.63	8.18 ± 0.34

[a] Cell viability was determined by the MTT assay after 24 h of treatment.

Esterification of WA with an α, β-unsaturated carboxylic acids and the biological evaluation of 8b-8l

Based on the above findings, we aimed to study the SAR by modifying the ketone to an α, β-unsaturated ketone. Compounds **8b-8l** (Scheme 3) were prepared with different acyl chlorides catalyzed by DMAP and EDCI in anhydrous CH_2Cl_2. The inhibitory activities against four cell lines, namely, HT-29, HCT-116, HepG2, and MCF-7, were evaluated. As shown Table 3, the inhibitory activity of almost all the substituent groups at the β position of the ketone were better than that of the natural product **WA**. The activity of **8b** with a methyl group at the β position was better than that of **8l** with a methyl group at the α position. Among these analogs, the compound with the most potential was **8e**, which contained an ester at the end.

Scheme 3. Synthesis of WA Analogs 8b-8l. [a]



[a] Reagents and conditions: DMAP, EDCI, Et_3N, anhydrous CH_2Cl_2, 0 ºC, 25h.

Table 3. Cytotoxic Activity (IC_{50} Values in μM) of 8a-8l against Four Cell Lines. [a]

Compd	R_1	R_2	Yield (%)	HT-29	HCT-116	HepG2	MCF-7
8a	H	H	71	0.78 ± 0.15	0.33 ± 0.07	0.52 ± 0.09	1.06 ± 0.12
8b	H	CH_3	60	0.37 ± 0.06	0.23 ± 0.05	0.30 ± 0.08	0.53 ± 0.09
8c	H	$(CH_3)_2$	68	0.46 ± 0.02	0.28 ± 0.02	0.26 ± 0.04	0.75 ± 0.11

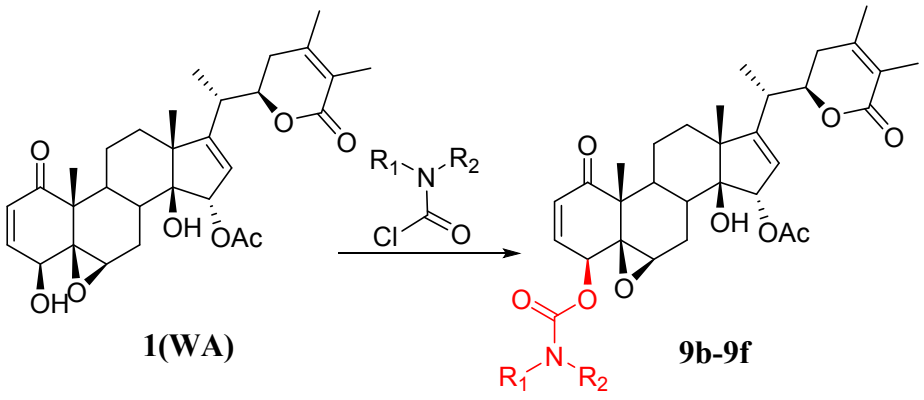
8d	H	$CH{=}CHCH_3$	74	1.42 ± 0.22	1.35 ± 0.09	1.52 ± 0.12	2.62 ± 0.13
8e	H	$COOCH_2CH_3$	79	0.23 ± 0.01	0.25 ± 0.08	0.24 ± 0.03	0.28 ± 0.04
8f	H	OCH_2CH_3	69	0.59 ± 0.07	0.67 ± 0.03	0.57 ± 0.05	1.21 ± 0.12
8g	H		71	0.45 ± 0.06	0.43 ± 0.03	0.31 ± 0.04	0.76 ± 0.17
8h	H		59	0.43 ± 0.10	0.38 ± 0.04	0.53 ± 0.03	0.63 ± 0.10
8i	H	Ph	37	0.77 ± 0.10	1.23 ± 0.06	0.70 ± 0.21	1.71 ± 0.19
8j	H	4-F-Ar	53	0.72 ± 0.02	1.03 ± 0.14	0.61 ± 0.08	1.58 ± 0.30
8k	H	$3\text{-}CF_3\text{-}Ar$	67	0.81 ± 0.25	0.65 ± 0.05	0.42 ± 0.01	1.39 ± 0.21
8l	CH_3	H	47	1.12 ± 0.17	1.34 ± 0.13	1.45 ± 0.12	2.11 ± 0.29

[a] Cell viability was determined by the MTT assay after 24 h of treatment.

Carbamate modification of WA and biological evaluation of 9b-9f

However, the carbamate group is very important in drug design and discovery. By introducing this bioactive pharmacophore to WA, **9a - 9f** (Scheme 4) were synthesized. The introduction of carbamate groups, regardless of whether the group was added to an opening alkyl group (**9a - 9c**) or to an alkyl ring (**9d - 9f**), improved the cytotoxic activity (Table 4) of the designed compounds. Among these compounds, compound 9c, with the ethyl carbamate, showed the best activity.

Scheme 4. Synthesis of WA Analogs 9b-9f. [a]



1(WA) **9b-9f**

[a] Reagents and conditions: DMAP, Et_3N, N_2, anhydrous CH_2Cl_2, 0 ºC, 12h.

Table 4. Cytotoxic Activity (IC_{50} Values in μM) of 9a-9f against Four Cell Lines. [a]

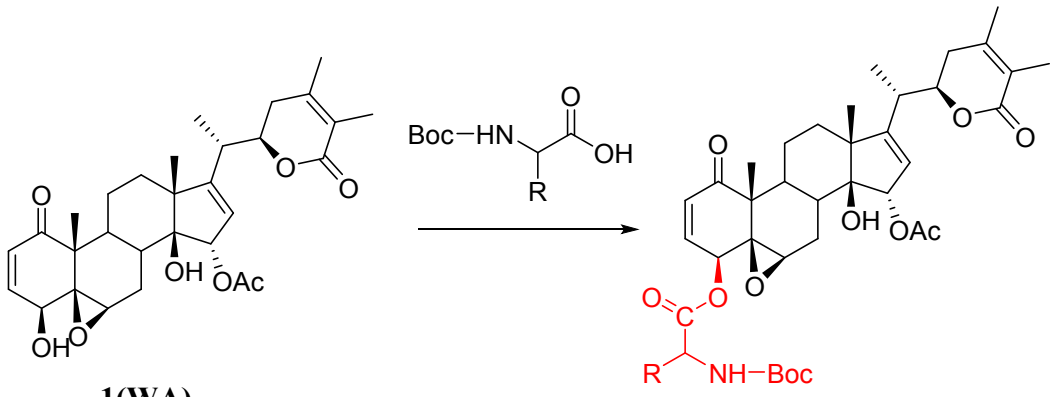
Compd	R_1	R_2	Yield (%)	HT-29	HCT-116	HepG2	MCF-7
9a	CH_3	CH_3	75	1.01 ± 0.08	0.67 ± 0.06	1.06 ± 0.14	2.10 ± 0.13
9b	CH_3	CH_2CH_3	70	1.78 ± 0.23	1.97 ± 0.15	2.37 ± 0.55	1.79 ± 0.14
9c	CH_2CH_3	CH_2CH_3	73	0.75 ± 0.17	0.43 ± 0.08	0.69 ± 0.12	1.21 ± 0.11
9d			52	1.12 ± 0.13	0.89 ± 0.09	1.23 ± 0.09	2.14 ± 0.23
9e			39	1.38 ± 0.04	0.73 ± 0.01	0.41 ± 0.01	4.31 ± 0.70
9f			42	0.99 ± 0.22	0.78 ± 0.11	0.67 ± 0.09	3.89 ± 0.63

[a] Cell viability was determined by the MTT assay after 24 h of treatment.

Amino group modification of WA and biological evaluation of 10a-10f

Amino groups are important building blocks in drug development and introducing amino acids into anticancer molecules can improve their selectivity to cancer cells, enhance the lipid solubility of drugs, and alleviate the toxicity of drugs to cells.[7] Compounds having amino groups, such as Docelaxel, Vancomycin, Polymyxin B, Daptomycin, 5-fluorouracil, etc., have superior anticancer activity compared with the original mother nucleus. Thus, amino acid groups were introduced in the modification of WA, and **10a - 10f** (Scheme 5) were synthesized. The biological evaluation indicated that amino group substitutions improved the cytotoxic activity of WA. Among these compounds (Table 5), **10e**, the Boc-Leu-modified WA analog showed the greatest potential.

Scheme 5. Synthesis of WA Analogs 10b-10f. [a]



1(WA) **10b-10f**

[a] Reagents and conditions: DMAP, EDCI, Et_3N, anhydrous CH_2Cl_2, 0 ºC, 25h.

Table 5. Cytotoxic Activity (IC_{50} Values in μM) of 10a-10f against Four Cell Lines. [a]

Compd	R	Yield (%)	HT-29	HCT-116	HepG2	MCF-7
10a	H	64	0.63 ± 0.04	0.47 ± 0.06	0.64 ± 0.09	2.16 ± 0.34
10b	CH_3	68	1.79 ± 0.04	1.95 ± 0.07	2.81 ± 0.11	4.33 ± 0.08
10c	$CH(CH_3)_2$	67	0.80 ± 0.07	0.60 ± 0.09	1.57 ± 0.15	3.05 ± 0.24
10d	$CH(CH_3)$ CH_2CH_3	71	1.08 ± 0.12	0.57 ± 0.11	1.66 ± 0.03	4.81 ± 0.11
10e	CH_2CH $(CH_3)_2$	73	0.78 ± 0.15	0.33 ± 0.07	0.52 ± 0.09	1.06 ± 0.12
10f		41	1.01 ± 0.08	0.67 ± 0.06	1.06 ± 0.14	2.10 ± 0.13

[a] Cell viability was determined by the MTT assay after 24 h of treatment.

Combined modifications and biological evaluation of 13a-13f

Given the above SAR studies, we concluded that the motif of an additional unsaturated ketone, which may act as a Michael addition receptor, and the alkyl carbamate groups improved the bioactivity of WA. Considering the bioisosterism principle of drug design in medicinal chemistry, a double bond was used to link the ketone and the carbamate groups together (Figure 2, Scheme 6). In accordance with this rule, we found that the bioactivity of all the analogs (Table 6) was not only stronger than that of WA but also stronger than that of the analogs with just one of the ketone

or the carbamate groups. Among **13a-13f**, **13a** displayed the highest potency against HT-29 cells with an IC_{50} value of 0.08 μM, which was more than a 30-fold increase compared to that of the starting compound **WA**. For the human normal cells, human normal hepatocytes L02 and human tubular epithelial cells HK-2, the experiment proved that WA is not so sensitive to the normal cells and the cancer cells as well in the same concentration (Table 7). But for **13a**, it is safe enough to normal cells when it is effective to the cancer cells at the concentration of 0.08 μM for HT-29, which is 66 times low for that of L02, and 56 times low for HK-2 (Table 7). The results show that **13a** contrast **WA** has better safety at effective concentration.

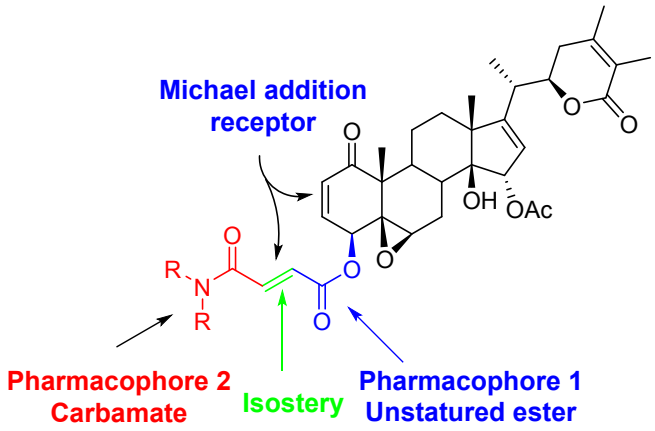
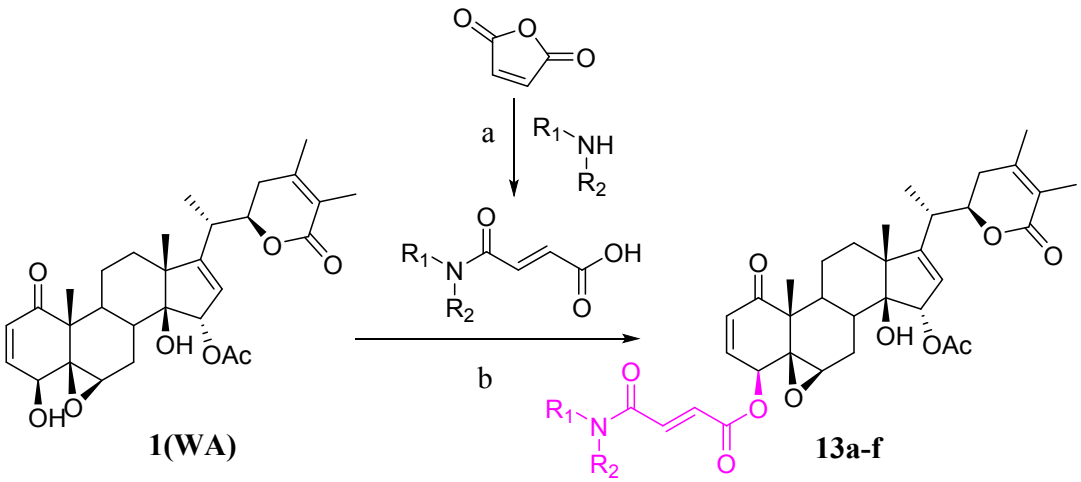


Figure 2. The combination design of the target compounds.

Scheme 6. Synthesis of WA Analogs 13a-13f. [a]



[a] Reagents and conditions: (a) Et_3N, anhydrous CH_2Cl_2, rt, 1h; (b) DMAP, EDCI, Et_3N, anhydrous CH_2Cl_2, 0 ºC, 25h.

Table 6. Cytotoxic Activity (IC_{50} Values in μM) of 13a-13f against Four Cell Lines. [a]

Compd	R_1	R_2	Yield (%)	HT-29	HCT-116	HepG2	MCF-7
13a	CH_3	CH_3	68	0.08 ± 0.02	0.13 ± 0.03	0.11 ± 0.01	0.33 ± 0.05
13b	CH_2CH_3	CH_2CH_3	56	0.30 ± 0.06	0.25 ± 0.02	0.31 ± 0.04	0.45 ± 0.06
13c	$CH(CH_3)_2$	$CH(CH_3)_2$	64	0.19 ± 0.04	0.35 ± 0.03	0.36 ± 0.05	0.62 ± 0.13
13d			47	0.25 ± 0.06	0.20 ± 0.04	0.29 ± 0.03	0.38 ± 0.02
13e			61	0.37 ± 0.02	0.16 ± 0.04	0.31 ± 0.05	0.61 ± 0.07
13f			70	0.43 ± 0.07	0.46 ± 0.07	0.37 ± 0.05	0.76 ± 0.15

[a] Cell viability was determined by the MTT assay after a 24 h treatment.

Table 7. Cytotoxic Activity (IC_{50} Values in μM) of WA, 13a and myricetin against six Cell Lines. [a]

Compd	L02	HK-2	HT-29	HCT-116	HepG2	MCF-7
WA	8.21± 0.76	6.41± 0.66	2.48 ± 0.23	2.37 ± 0.22	2.85 ± 0.12	6.32 ± 0.74
13a	5.25± 0.49	4.53± 0.37	0.08 ± 0.02	0.13 ± 0.03	0.11 ± 0.01	0.33 ± 0.05
myricetin	> 20.00	> 20.00	> 20.00	> 20.00	> 20.00	> 20.00

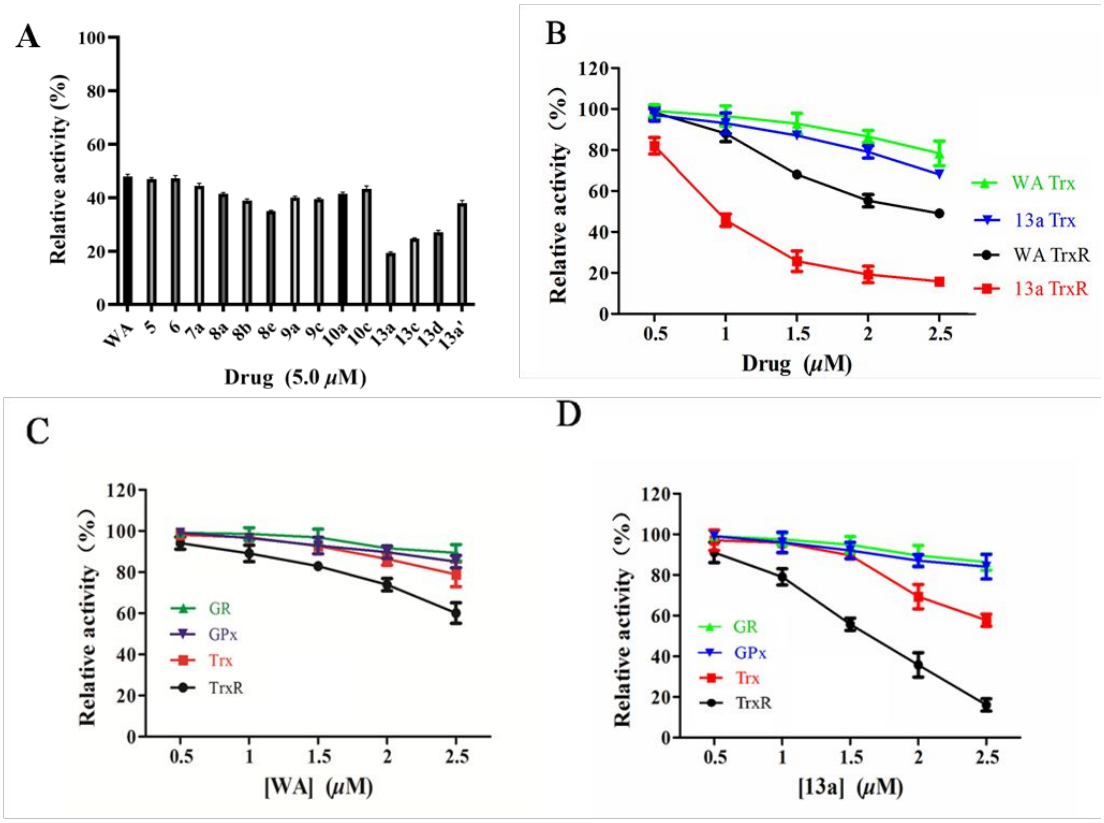
[a] Cell viability was determined by the MTT assay after a 24 h treatment.

Inhibition of TrxR in both cell-free and cell-based assays

After several SAR studies, **13a** was shown to display the highest cytotoxicity against the four cancer cell lines; thus, we addressed the possible cellular target of the compound. The expression of TrxR in four cancer cells and two normal cells was detected using Western blot **(**Figures **S2)**. The results showed that the TrxR expression levels of HT-29, HCT-116 and HepG2 were much higher than those of MCF-7, L02 and HK-2 cells. Meanwhile, the cytotoxic activity of WA and its

derivatives against several kinds of cells was tested and suggested that the cells with high expression of TrxR were more active than those with low expression levels (Table 7). The core feature of **WA** is the presence of an α,β-unsaturated ketone moiety in ring A, which is the key structural motif of many reported TrxR inhibitors.[28-30] We speculated that **13a** might be a novel inhibitor of TrxR after using PharmMapper to screen targets of the potential protein (Figures **S3**).[31] A DTNB reduction assay was used to determine the activity of TrxR. First, compounds with good cytotoxic activity were selected for preliminary screening of TrxR inhibitory activity, as a result, **13a** showed the best inhibitory activity on TrxR (Figure 3A). Then, in vitro experiments on enzymes were performed. As shown in Figure 3B, WA clearly inhibited TrxR with an IC_{50} value of approximately 2.64 μM (line with closed squares), and **13a** clearly inhibited TrxR with an IC_{50} value of approximately 0.85 μM (line with closed squares). Trx was weakly inhibited by both compounds (IC_{50} > 2.50 μM), suggesting that **WA** and **13a** inhibited TrxR much more than Trx. We then examined the relationship of the compounds to some intracellular (HT-29) enzymes involved in oxidative stress. Glutathione reductase (GR) is a homolog of TrxR with a similar overall structure. Like TrxR, glutathione peroxidase (GPx) is a Sec-containing enzyme. Both GR and GPx are closely related to ROS. As shown in Figures 3C and 3D, the inhibition of TrxR induced by **WA** and **13a** was much higher than that of the other enzymes in HT-29 cells. The TrxR inhibition activity was tested in 30 min, 60 min and 90 min, respectively (Figure 3E). Comparing the inhibitory activity of compound **8a**, which did not have dimethylformamide group, the TrxR inhibitory activity of **13a** is ten times stronger than that of **8a.** It means that the dimethylformade group is the necessary bioactivity group for inhibiting TrxR. When the double bond of the acrylamide group in **13a** is reduced as in **13a'** (Figures **S4)**, the TrxR inhibition bioactivity dropped three to four times. It suggested that the double bond is the indispensable group contributing to the

TrxR inhibition activity. All the results indicated that **13a** had stronger inhibitory activities than did **WA** inside and outside the HT-29 cells. Taken together, the results indicated that **13a** selectively targeted and inhibited TrxR in both cell-free and cell-based assays.



E

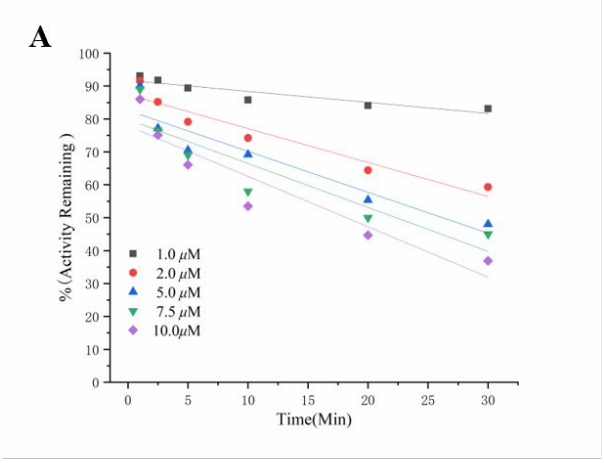
Compd	TrxR(30min) (μM)	TrxR(60min) (μM)	TrxR(90min) (μM)
WA	4.92±0.16	3.35±0.11	2.64±0.07
8a	3.91±0.13	2.85±0.04	2.06±0.09
13a	0.97±0.05	0.87±0.06	0.85±0.04
13a'	3.81±0.14	2.91±0.16	2.15±0.06
myricetin	0.93±0.08	0.72±0.05	0.65±0.04

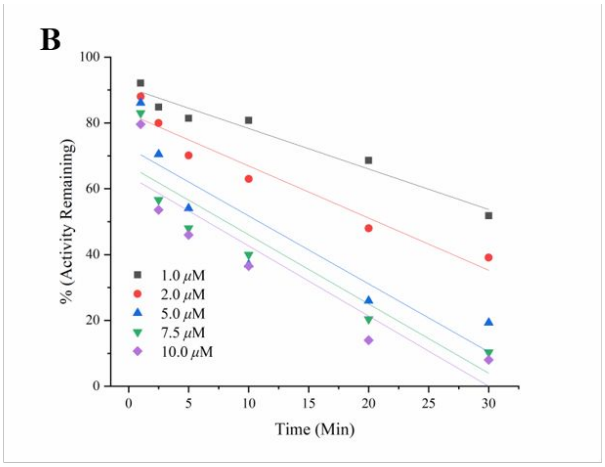
Figure 3. Enzyme and protein inhibition. All activity values are expressed as the percentage of the control. (A) TrxR inhibition activity by the compounds at 5.0 μM. (B) Inhibition of TrxR and Trx induced by **WA** and **13a**. (C) Inhibition of TrxR, Trx, GR and GPx induced by WA in HT-29 cells.

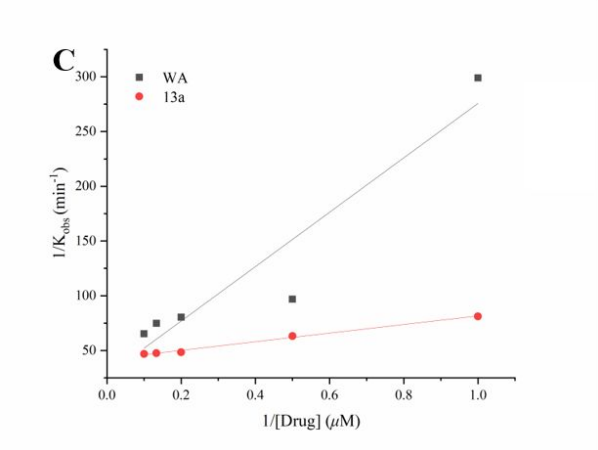
(D) Inhibition of TrxR, Trx, GR and GPx induced by **13a** in HT-29 cells. (E) Inhibition of TrxR by different incubation time (30min, 60min, 90min).

K_{inact} and K_i

The reaction kinetic parameters including the covalent bond formation (K_i), the rate constant for irreversible inactivation (k_{inact}) was determined and shown in Figure 4. Comparing with the K_i of **WA, 13a** has higher affinity, which is about 10 times of **WA**. For the K_{inact} of **13a** and **WA**, it showed that **13a** had better covalent response capacity than **WA.** In general, the kinetic parameters showed that **13a** is a better covalent bond inhibitor of TrxR than **WA**.







D

Compd	K_i (μM)	k_{inact} (min^{-1})	k_{inact}/K_i ($\mu M^{-1}min^{-1}$)
WA	9.117	0.0367	0.0040
13a	0.932	0.0237	0.0254

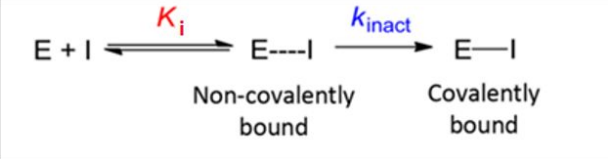
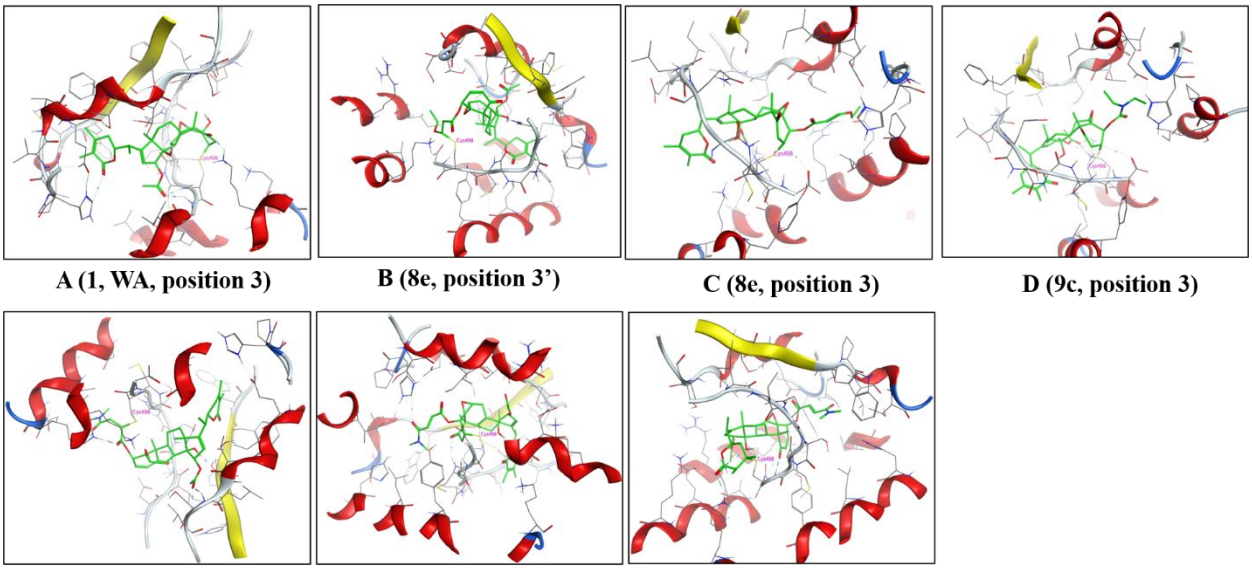


Figure 4. The kinetics of the inhibitors **WA** and **13a** to **TrxR**. (A) The reactive velocity of **WA** on TrxR. (B) The reactive velocity of **13a** on TrxR. (C) Double-reciprocal plot of K_{obs} and concentration of **WA** and **13a**. (D) Ki and K_{inact} of **WA** and **13a on** TrxR.

Molecular modeling. Since **13a** showed relatively good TrxR inhibitory activity, molecular docking experiments of **13a** and the starting compound WA were conducted to study whether there was a direct interaction between the compound and TrxR. In addition, **8e** and **9c**, the compounds with the highest potential in their respective series, were included in a docking study to interpret the interaction mechanisms between the designed compounds and TrxR. A covalent docking study is performed using Schrödinger software. The crystal structure of rat TrxR1 was obtained from the Protein Data Bank (PDB ID: 3EAN). Given Michael reaction mechanics, Sec498 is believed to be the Michael donor.[32,33] Therefore, we initially used Sec498 as the binding residue to perform covalent docking. However, a persistent error remained when Sec was used for docking because Sec is not a standard amino acid. Therefore, we mutated Sec to Cys on the basis that Sec is a cysteine analog with a selenium-containing selenol group in place of the sulfur-containing thiol group, which will have a minute influence on the docking result. The molecular docking results and the docking interaction methods are presented in Figure 5, Figure S5 and Table S2, respectively. Through the combined analysis of the docking score and the cytotoxic activity, the following two essential conclusions were reached. One conclusion is that the Michael addition can take place at the β-position of the α,β-unsaturated carbonyl in ring A of WA (position 3; Figures 5A, 5C, 5D and 5F), the β-position of the α,β-unsaturated carbonyl in the side chain of 4-OH (position 3', Figures 5B and 5E) and the α-position of the α,β-unsaturated carbonyl in the side chain of 4-OH (position 2', Figure 5G). The other conclusion is that the interaction with the epoxy group is important (Table S2); for example, in **1** from Figure 4A, A494 interacts with the epoxy,

and Cys498 interacts with **8e** (Figure 5B). Moreover, Cys498 interacts with **13a** (Figure 5E), which is in agreement with the classic SAR studies that epoxy at positions 5 and 6 is an essential group for the bioactivity of withanolides.[24] These basic conclusions indicate that the α, β-unsaturated chain at 4-OH from compound **13a** is necessary. The Michael donor, residue Cys498, is preferred to react with the α, β-unsaturated chain at 4-OH assisted by the reaction between the epoxy and the amine from Cys498. Once the covalent bond formed, which pushed the whole **13a** inward to the gap between chains A and B, the inner residue A472 pulled the 14-OH group via hydrogen bonding interactions (Figure 5E, Table S2). In contrast, when Michael addition took place at position 3 or 2', the corresponding interactions could not form, and the docking scores of the compounds were smaller, especially at position 2' (docking score: -3.816), than those of the compounds in which docking occurred at position 3' (docking score: -5.614). Thus, **13a** has a strong binding interaction with TrxR through a covalent bond, which is consistent with the cytotoxic activity studies.



A (1, WA, position 3) **B (8e, position 3')** **C (8e, position 3)** **D (9c, position 3)**

E (13a, position 3') **F (13a, position 3)** **G (13a, position 2')**

Figure 5. The covalent docking adducts of TrxR (PDB: 3EAN mutated Sec498 to Cys498) and **1 (A)**, **8e (B and C)**, **9c (D)** and **13a (E, F and G).** The number in the brackets represent the position in Figures 2 and 4 on the compounds where Michael addition occurred.

Identification of the covalent adduct between 13a and TrxR. Molecular docking suggested that **13a** might modify the reduced enzyme by forming a covalent adduct with the nascent selenol/thiol of the active site. To confirm this hypothesis, we preincubated the reduced enzyme with a large excess of **13a** (20-fold) and then digested the protein with trypsin. By analyzing the mass of the peptides, we identified the modified residue. Usually, TrxR will produce the classic reactive peptides WGLGGTCVNVGCIPK (53-67), RSGGDILQSGCUG (487-499) and SGGDILQSGCUG (488-498), and these peptides were identified by the protein mass of the control, as shown in Table 8 and Figure 6. Figure 6A shows the control tryptic ion at *m/z* 571.6876, with distinctive isotope patterns and confirmed by the critical *b* and *y* ions, which were identified as a doubly charged ion of the SGGDILQSGCUG peptide (residues 488−499 in TrxR). The MS^1 of the peptide SGGDILQSGCUG (residues 488−499 in TrxR) was identified through a single charged ion with *m/z* 1142.3667 (Figure 6B) and a doubly charged ion with *m/z* 571.6870 (Figure 6C). Figure 6D presents the MS spectrum of CID products from the control tryptic ion at *m/z* 649.7377, which was identified as a doubly charged ion of the RSGGDILQSGCUG peptide (residues 487−499 in TrxR). In the reaction sample, we identified adducts of **13a** and the peptide RSGGDILQSGCUG or SGGDILQSGCUG. The tryptic ion at *m/z* 976.4026 in Figure 6E was identified as a doubly charged ion with one **13a** (MW, 651.75) adducted to the RSGGDILQSGCUG peptide (MW, 1297.47, residues 487−499 in TrxR). In Figure 6F, the MS/MS spectrum of CID products from the tryptic ion at *m/z* 898.4107 is shown. This ion with distinctive isotope patterns was identified as a doubly charged ion with one **13a** adducted to the

SGGDILQSGCUG peptide (1141.37), and **13a** was bound to selenocysteine 498 via the Michael addition reaction, which was undoubtedly confirmed by the critical *b* and *y* ions (Figure 6F).

Table 8. Modified Peptides in the Tryptic Digest of 13a-Treated TrxR

peptide sequence	*m / z*	charge	t_R (min)	$[M + H]^+$	sample
WGLGGTCVNVGCIPK	752.3722	2	38.76	1503.7371	control
SGGDILQSGCUG	571.6876	2	32.67	1142.3679	control
SGGDILQSGCU-**13a**G	898.4107	2	31.71	1795.8141	reaction
RSGGDILQSGCUG	649.7374	2	28.63	1298.4675	control
RSGGDILQSGCU-**13a**G	976.4026	2	33.89	1951.7979	reaction

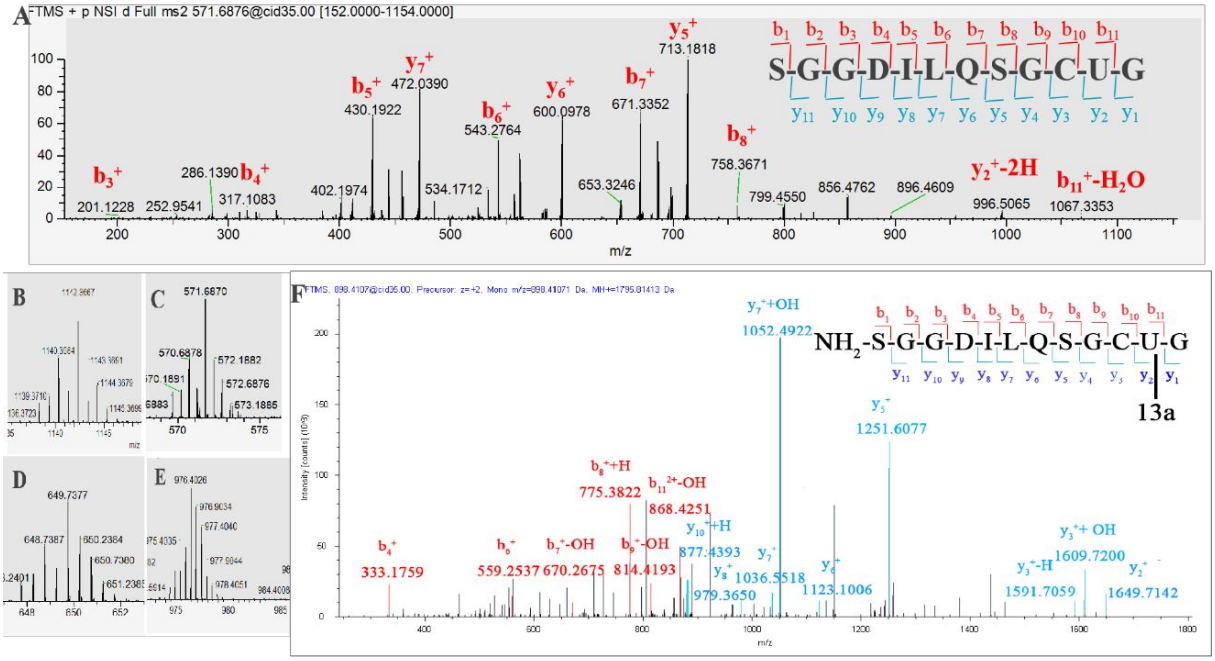


Figure 6. LC-MS/MS analysis of control peptides (A-D) and the **13a**-TrxR adducted tryptic peptide (E, F).

The binding ability of 13a to sulfhydryl groups. Intracellular Cys residues are prone to Michael addition reactions with α, β-unsaturated ketones. To examine the ability of the compounds to bind to sulfhydryl groups, we conducted in vitro binding experiments (Figure S6).

The reaction products of **13a** and NAC were analyzed using online NMR spectroscopy (Figure S6). The experimental results showed that the double bonds at positions 2 and 3 from the A ring were easier to react with SH on NAC than the double bonds at positions 2' and 3' from the side chain. Meanwhile, the reaction products of **WA**, **13a** with NAC and DTT were also detected by LC-MS. The results showed that both WA and 13a readily react with DTT and NAC, the standard test reagents used for the detection of sulfhydryl groups (Figure S7). Next, we examined the effect of the compounds on the total amount of sulfhydryl groups in HT-29 cells. As shown in Figure S6D, with increasing compound concentrations, the total content of sulfhydryl groups in cells decreased gradually, and the effect of 13a was much stronger than that of WA. This finding suggests that the compounds can directly or indirectly reduce the total amount of sulfhydryl groups in HT-29 cells.

Effects of 13a on ASK-1 and P53 signaling. We determined the impact of **13a** on the expression of TrxR and Trx in HT-29 cells by Western blot analysis. As shown in Figure 7A, **13a** significantly reduced the relative levels of TrxR and Trx expression in HT-29 cells. TrxR inhibitory activity can destroy the redox function of some cells, leading to apoptosis signaling and regulating the activation of a kinase (ASK1). The reduced Trx binds to the Cys residues of ASK1, inhibiting its kinase activity.[34] As shown in Figure 7A, **13a** significantly reduced the ASK1 to p-ASK1 expression ratio in HT-29 cells. Trx can affect the oxidation/reduction state of key Cys residues in the DNA binding domain of the human cancer suppressor gene (P53), thereby affecting

the ability of P53 to bind to DNA. When cancer cells use the Trx system to fight apoptosis, this process may be accompanied by the inactivation of the P53 system, thus enhancing the viability of cells.[35] **13a** significantly increased the relative levels of P53 to GAPDH expression in HT-29 cells. Hence, the decrease in the levels of TrxR and Trx expression induced by **13a** may contribute to the TrxR inhibitory activity and anticancer activity of **13a** in cancer cells.

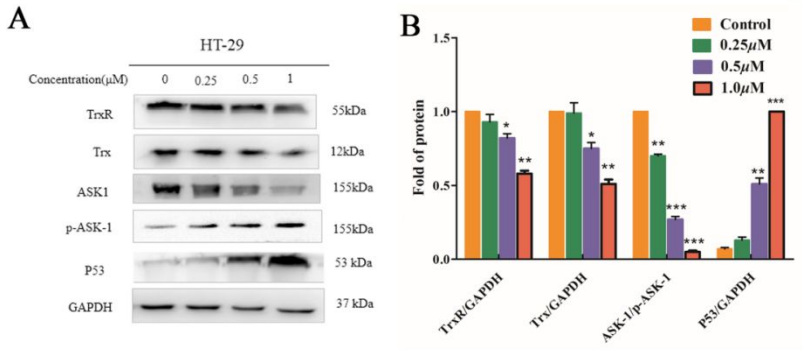


Figure 7. (A) Effect of **13a** on the relative levels of TrxR, Trx, ASK-1, p-ASK-1 and P53 expression in HT-29 cells. Cells were treated with **13a** or vehicle for 24 h, and the levels of TrxR, Trx, ASK-1, p-ASK-1 and P53 relative to the expression of the control GAPDH were determined by Western blot analysis. Data are representative of three independent experiments. *P < 0.05, **P < 0.01 vs the control.

Effect of 13a on ROS accumulation in cells. The TrxR system is a vital component involved in the maintenance of intracellular redox homeostasis and in defense against oxidative stress. TrxR is the only known enzyme that maintains reduced Trx pools for ribonucleotide reductase in DNA synthesis[36] and the pools of many antioxidant enzymes, such as peroxiredoxins[37] and Met sulfoxide reductases,[38] under physiological conditions. The inhibition of TrxR can disturb the cellular redox balance and cause the accumulation of ROS in cells. DCFH-DA is a well-established probe used to detect the intracellular production of ROS. After being taken up by cells, DCFH-DA is hydrolyzed by cellular esterases into dichlorodihydrofluorescin (DCFH), which is trapped within

the cell. The nonfluorescent DCFH is then oxidized to fluorescent dichlorofluorescin by the action of cellular ROS. In our experiments (Figure 8), the level of ROS in HT-29 and HCT-116 cells was dose-dependently increased by **13a** treatment. Pretreatment with 5 mM GSH, an ROS scavenger, could weaken the compound-induced ROS production in two types of cells. In addition, pretreatment with 5 mM GSH rescued two types of cells from the cytotoxic effects of **13a**, suggesting that the antiproliferative effect of **13a** was ROS dependent.

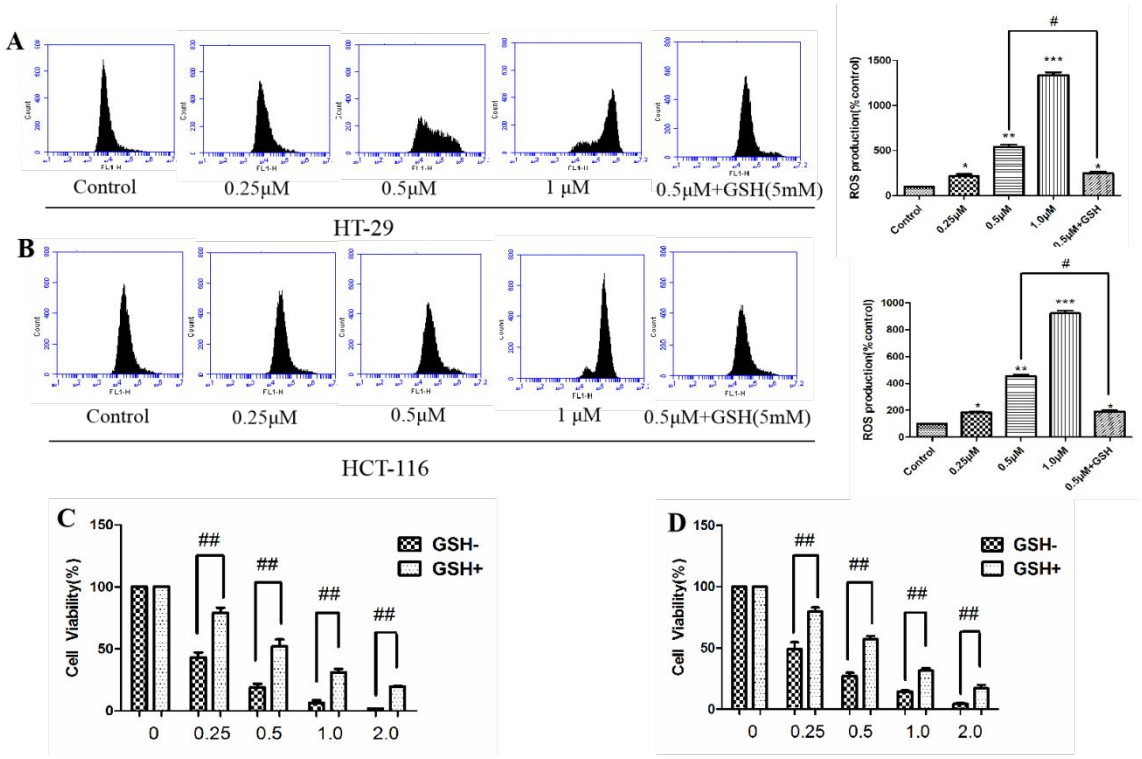


Figure 8. Effect of **13a** on ROS accumulation in cells. (A) HT-29 cells were incubated with the indicated concentrations of **13a** for 24 h. The ROS level was measured by flow cytometry. (B) HCT-116 cells were incubated with the indicated concentrations of **13a** for 24 h. The ROS level was measured by flow cytometry. (C) HT-29 cells were treated with the indicated concentrations of **13a** alone or in combination with the ROS scavenger GSH (5 mM), and cell viability was determined by MTT assay. (D) HCT-116 cells were treated with the indicated concentrations of

13a alone or in combination with the ROS scavenger GSH (5 mM), and cell viability was determined by MTT assay.

Monoclonal formation experiment. Cloning experiments are an effective method to determine the proliferative capacity of a single cell. To test the effect of 13a on the ability of HT-29 cells to form monoclonal cells, we treated cells with different concentrations of **13a** for 24 h. After treatment administration, the normal culture medium was changed daily for 14 days. The results (Figure 9A) showed that the number of clones in the administration group was significantly reduced compared with that in the DMSO control group, showing the concentration-dependent inhibition of HT-29 cell monoclonal ability by **13a**. The study demonstrated that **13a** can inhibit the proliferation of colon cancer cells.

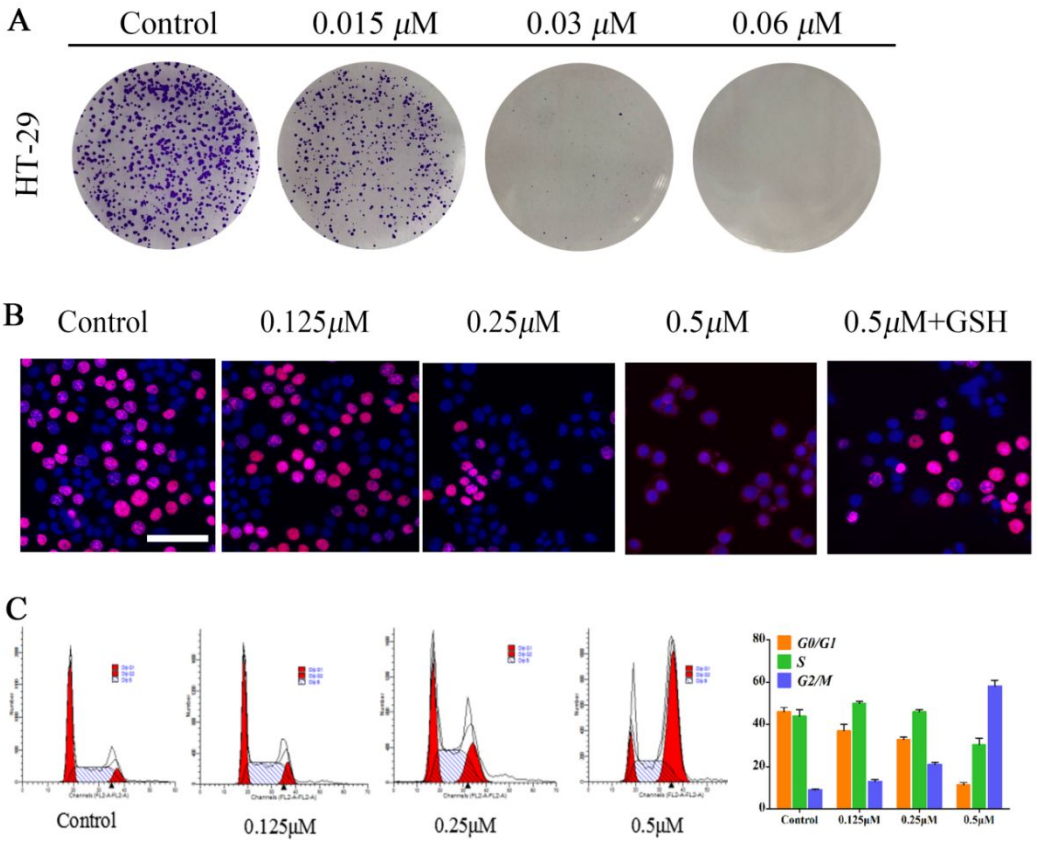


Figure 9. Effect of **13a** on the proliferation of HT-29 cells. (A) The effect of **13a** on the ability of HT-29 cells to form monoclonal cells. (B) HT-29 cells were incubated with various concentrations of **13a** alone or in combination with the ROS scavenger GSH (5 mM) for 24 h. EdU staining was then performed, and the cells were observed by confocal microscopy. Blue cells were counted as Hoechst-positive cells; red cells were counted as EdU-positive cells. Scale bar: 20 μm. (C) HT-29 cells were treated with the indicated concentrations of **13a** for 24 h, and the distribution of the cell cycle was monitored by flow cytometry. Columns showing the percentage of HT-29 cells in the G0/G1, S and G2/M phases of the cell cycle.

EdU infiltration experiment. Next, an EdU kit was used to further verify the effect of the drug on cell proliferation. EdU is a thymine nucleoside analog that replaces T during cell proliferation to infiltrate into the DNA that is being copied. We applied different concentrations of **13a** to HT-29 cells, and the number of EdU-positive cells was significantly lower in the treatment group than in the control group (Figure 9B), which indicates that **13a** can effectively inhibit the proliferation of cancer cells.

Induced G2/M arrest in HT-29 cells. To learn more about the antiproliferative mechanism of **13a**, the effect of **13a** on cell cycle progression was explored using flow cytometric analysis after propidium iodide (PI) staining (Figure 9C). The results showed that the number of cells in the G2/M fraction increased significantly and dose-dependently after 24 h of exposure to **13a**. The cell cycle distribution is summarized in the histograms shown in Figure 8C, and the results suggest that **13a** induced G2/M phase arrest in HT-29 cells, which was consistent with reports that **13a** induced G2/M arrest.

Induction of HT-29 cell apoptosis. To determine whether the inhibitory effects of **13a** on colon cancer cell proliferation were accompanied by enhanced cancer cell apoptosis, Annexin V-FITC

and PI staining were carried out, and the percentages of apoptotic cells were tested using a flow cytometry assay. HT-29 cells were incubated with different concentrations of compound **13a** for 24 h. The results (Figure 10A) show that **13a** mainly induced apoptotic cell death in HT-29 cells. Furthermore, a Western blot assay (Figure 10C) revealed that treatment with **13a** dramatically decreased the expression levels of antiapoptotic Bcl-2 but increased the expression levels of proapoptotic Bax in HT-29 cells. Quantitative analysis revealed that treatment with **13a** significantly increased the ratio of Bax to Bcl-2. Caspase activation is a critical event in the initiation and execution of apoptosis in cells. Treatment with **13a** also significantly increased the relative levels of cleaved-caspase-3 and poly ADP-ribose polymerase (PARP) in HT-29 cells.

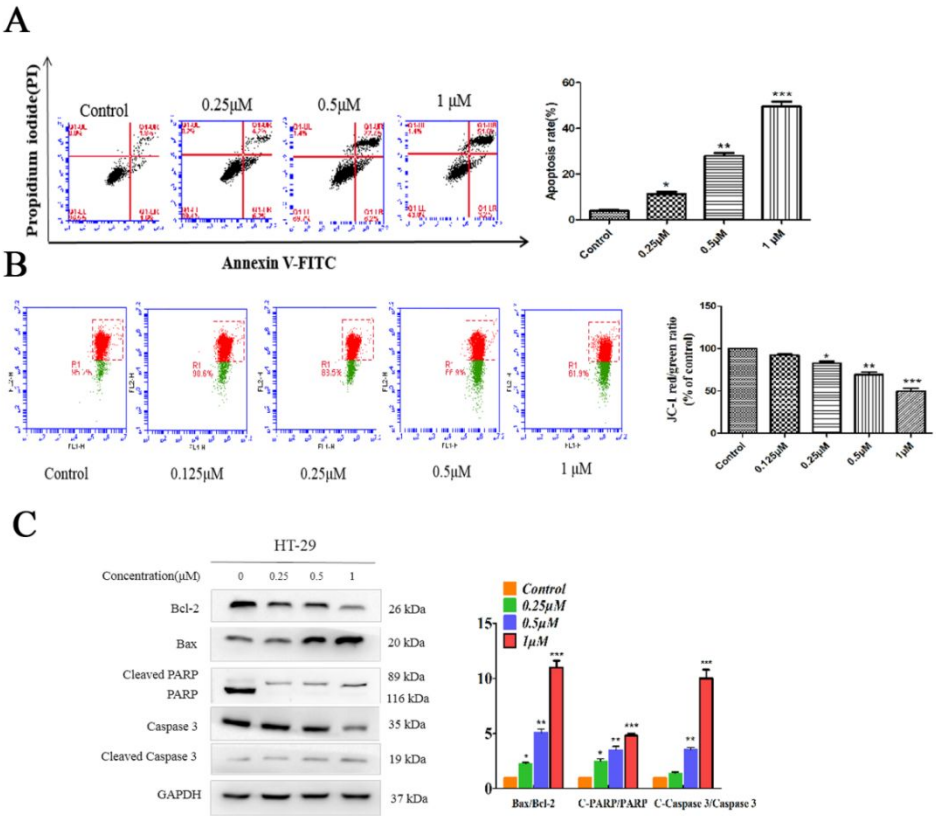


Figure 10. Effect of **13a** on apoptosis induction in HT-29 cells. (A) HT-29 cells were incubated with the indicated different concentrations of **13a** for 24 h and stained with Annexin V-APC and 7-AAD. (B) HT-29 cells were incubated with the indicated concentrations of **13a** for 24 h and

stained with JC-1. (C) Western blot analysis was performed with antibodies specific for Bcl-2, Bax, caspase-3, cleaved-caspase-3, PARP, and cleaved-PARP, and GAPDH was used as the loading control.

Effect of 13a on the mitochondrial membrane potential of cells. Above, we demonstrated that **13a** significantly increased the ratio of Bax to Bcl-2. An increased ratio of Bax/Bcl-2 protein can promote mitochondrial membrane depolarization and induce apoptosis. Many studies have shown that mitochondrial dysfunction is closely related to apoptosis and plays an important role in the apoptosis pathway. Therefore, we examined whether the drug can cause mitochondrial damage. JC-1 is a cationic lipid fluorescent dye and is usually used to detect mitochondrial membrane potential. The changes in mitochondrial membrane potential after administration were detected by flow cytometry. The results showed that, with increasing **13a** concentrations, the ratio of red fluorescence to green fluorescence relative to the blank control group decreased (Figure 10B). This finding indicated that **13a** can reduce the mitochondrial membrane potential of HT-29 cells.

CONCLUSIONS

In summary, we designed 41 semisynthetic analogs of WA. Iterative SAR studies of the target compounds were performed. Among all the compounds, **13a** displayed the highest cytotoxicity toward HT-29 cells (IC_{50} = 0.08 μM), which is approximately 30 times greater than the initial natural product WA (IC_{50} = 2.48 μM). Furthermore, **13a** suppressed TrxR expression through covalent bonding to 498 amino acid residues by Michael reaction, as demonstrated by protein MS/MS and molecular docking. As a result, **13a** promoted intracellular ROS accumulation and decreased the level of reduced Trx by changing the expression of ASK1 and P53; moreover, **13a**

exerted an antiproliferation effect by inducing apoptosis and cell cycle arrest (Figure 11). **13a**, as novel TrxR inhibitor, may be a promising candidate for cancer intervention.

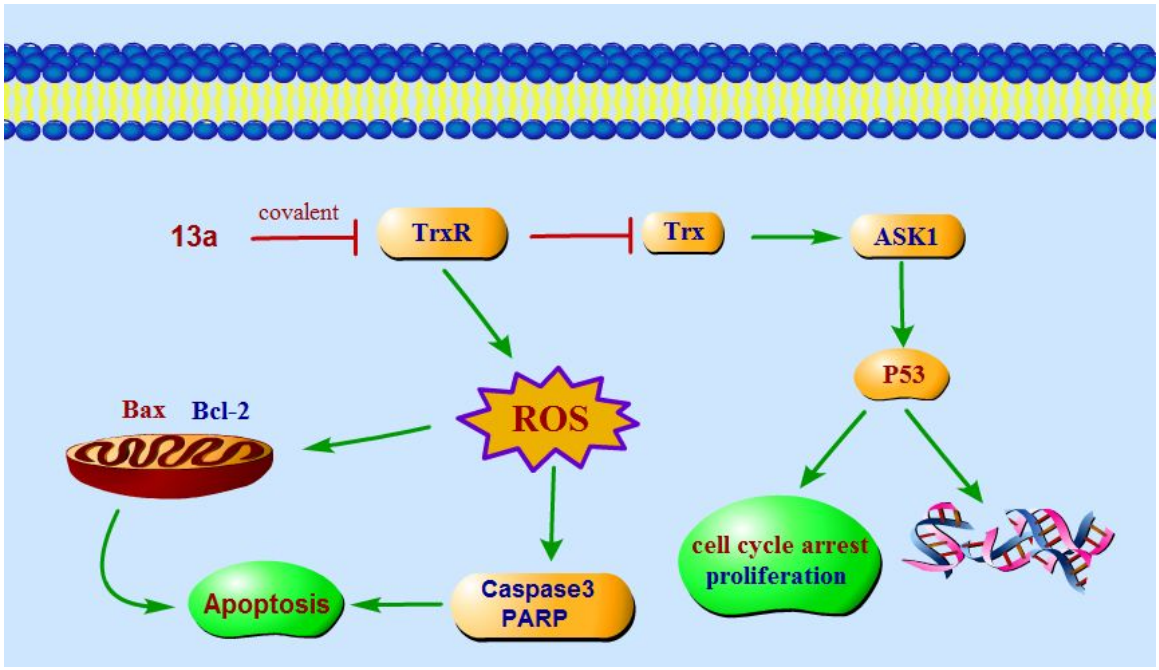


Figure 11. Summary of the physiological activity of **13a** in HT-29 cells.

EXPERIMENTAL SECTION

Chemistry

All reagents were purchased from Sigma-Aldrich, Energy Chemical, Aladdin or Macklin. Thin layer chromatography (TLC) was performed using silica gel-coated aluminum plates with fluorescent indicators and was visualized with ultraviolet light at 254 nm. Melting points were measured on an XT-4 digital display micromelting point apparatus (Taike, Beijing, China), uncorrected. ^{1}H NMR spectra were recorded on a Bruker Ultrashield 500 Plus NMR spectrometer, and ^{13}C NMR spectra were recorded on a Bruker Ultrashield 125 Plus NMR spectrometer. Chemical shift values (δ) were expressed as parts per million (ppm) relative to tetramethylsilane as the internal standard. High-resolution electrospray ionization (HRESI) mass spectra were carried out using an Agilent 6520B Q-TOF mass spectrometer (Agilent Technologies, Santa Clara,

CA, USA). High performance liquid chromatography (HPLC) was performed for purity checking using Agilent 1100 series HPLC on a Shimadzu XDB-C18 (5 μm, 4.6 × 150 mm^2) column. All the final compounds were found to be pure up to 95% or higher.

Isolation of Naturally Occurring Withangulatin A (1) Withangulatin A (1) was obtained from the whole herb of *Physalis angulata* L.[19] White powder; m.p. 151-152 ºC; ^{1}H NMR (500 MHz, $CDCl_3$) δ 6.95 (dd, J = 10.0 and 6.0 Hz, 1H, H-3) , 6.18 (d, J = 10.0 Hz, 1H, H-2), 5.69 (d, J = 5.0 Hz, 1H, H-16), 5.23 (d, J = 5.0 Hz, 1H, H-15), 4.23 (m, 1H, H-22), 3.82 (d, J = 2.5 Hz, 1H, H-4), 2.59 (dt, J = 14.0 and 3.0 Hz, 1H, H-7α), 2.50 (m, 1H, H-20), 2.40 (t, J = 16.0 Hz, 1H, H-23β), 2.17 (dd, J = 16.0 and 3.5 Hz, 1H, H-23α), 1.95 (s, 3H, OAc-15), 1.93 (s, 3H, H-28), 1.86 (s, 3H, H-27), 1.82-1.50 (m, 7H, H-7β, H-8, H-9, H-11, H-12), 1.43 (s, 3H, H-19), 1.12 (d, J = 6.0 Hz, 3H, H-21), 1.10 (s, 3H, H-18); ^{13}C NMR (125 MHz, $CDCl_3$) δ 202.7 (C-1) , 169.9 (C-15-O$\underline{C}CH_3$), 166.2(C-26), 162.6(C-17), 148.6(C-24), 142.67(C-3), 131.6(C-2), 122.4(C-25), 121.1(C-16), 83.7(C-15), 81.6(C-14), 79.4(C-22), 69.7(C-4), 63.7(C-5), 63.3(C-6), 52.2(C-13), 47.7(C-10), 39.3(C-9), 37.6(C-12), 35.5(C-20), 34.7(C-8), 33.1(C-23), 24.8(C-7), 22.1(C-11), 21.5(C-15-OC$\underline{C}H_3$), 20.6(C-28), 17.7(C-18), 17.6(C-21), 16.1(C-19), 12.6(C-27); HRMS: $[M + Na]^+$ calcd for $C_{30}H_{38}O_8Na$ 549.2459, found 549.2463; purity 95.78% (MeOH/H_2O = 75/25, R_t = 3.029 min).

General Procedure for the Synthesis of 2−12.

(2a). To a solution of the **1** (26.3 mg) and sarcosine (8.9 mg) in anhydrous CH_2Cl_2, and the mixture was stirred at 25 °C for 2h. **2a** was obtained as a white powder, 26.1 mg, yield 85 %; m.p. 163-164 ºC; ^{1}H NMR (500 MHz, $CDCl_3$) δ 5.71 (d, J = 5.0 Hz, 1H), 5.24 (d, J = 5.0 Hz, 1H) ,4.23 (m, 1H), 3.62 (s, 3H), 3.32 (s, 1H), 3.13 (dd, J = 12.0 Hz, 5.0 Hz, 1H), 2.86 (m, 1H), 2.69 (s, 1H), 2.59-2.45 (m, 7H), 2.40 (t, J = 16.0 Hz, 1H), 2.19 (dd, 16.0 Hz, 3.5 Hz, 1H), 2.04 (s, 3H), 1.93 (s, 3H), 1.86 (s, 3H), 1.82-1.50 (m, 8H), 1.37 (s, 3H), 1.13 (d, J = 6.0 Hz,3H), 1.07 (s, 3H); ^{13}C NMR

(125 MHz, $CDCl_3$) δ 211.2, 169.8, 166.3, 162.6, 148.6, 122.4 121.0, 83.8, 81.6, 79.5, 73.7, 67.2, 64.8, 63.7, 62.6, 52.3, 50.5, 50.5, 38.2, 37.4, 36.0, 35.5, 34.2, 33.1, 24.9, 21.8, 21.6, 20.6, 17.7, 16.8, 16.1, 12.6; HRMS: $[M + Na]^+$ calcd for $C_{33}H_{45}NNaO_{10}$ 638.2935, found 638.2936; purity 96.16% ($MeOH/H_2O$ = 40/60-80/20, R_t = 3.147 min).

(2b). To a solution of the **1** (26.3 mg), and imidazole (3.4 mg) anhydrous CH_2Cl_2, and the mixture was stirred at 25 °C for 2h. **2b** was obtained as a faint yellow powder, 25.3 mg, yield 85 %; m.p. 157-159 °C; ^1H NMR (500 MHz, $CDCl_3$) δ 7.54 (s, 1H),7.26 (s, 1H), 7.08 (s, 1H), 5.73 (d, J = 5.0 Hz, 1H) ,5.18 (d, J = 5.0 Hz, 1H) ,4.73 (m, 1H), 4.23 (m, 1H), 3.61 (s, 3H), 3.46 (dd, J = 12.0 Hz, 5.0 Hz, 1H), 3.10 (s, 1H), 2.87 (m, 1H), 2.59-2.45 (m, 7H), 2.09 (dd, 16.0 Hz, 3.5 Hz, 1H), 1.93 (s, 3H), 1.85(s, 3H), 1.82-1.50 (m, 8H), 1.46 (s, 3H), 1.12 (d, J = 6.0 Hz, 3H), 1.09 (s, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 209.8, 169.9, 166.5, 162.6, 148.7, 122.3, 121.0, 117.3, 84.0, 81.5, 79.7, 63.0, 61.6, 56.2, 53.6,52.2, 50.8, 39.7, 38.7, 37.5, 35.5, 34.1, 33.2, 29.9, 24.5, 22.0, 21.6, 20.6, 17.9, 16.9, 16.1, 12.6; HRMS: $[M + Na]^+$ calcd for $C_{33}H_{42}N_2O_8Na$ 617.2833, found 617.2836; purity 95.15% ($MeOH/H_2O$ = 40/60-80/20, R_t = 4.535 min).

(2c). To a solution of the **1** (26.3 mg), and HCl solution in ether anhydrous CH_2Cl_2, and the mixture was stirred at 25 °C for 1h. **2c** was obtained as a faint yellow powder, 16.7 mg, yield 60 %; m.p. 147-149 °C; ^1H NMR (500 MHz, $CDCl_3$) δ 6.59 (dd, J = 10.0 Hz, 6.0 Hz, 1H), 5.93 (d, J = 10.0 Hz, 1H), 5.69 (d, J = 5.0 Hz, 1H), 5.31 (d, J = 5.0 Hz, 1H), 5.17 (s, 1H), 4.72 (s, 1H), 4.23 (m, 1H), 2.59 (dt, J = 14.0 Hz, 3.0 Hz, 1H), 2.50 (m, 1H), 2.40 (t, J = 16.0 Hz, 1H), 2.17 (dd, J = 16.0 Hz, 3.5 Hz, 1H) 2.02 (s, 3H), 1.95 (s, 3H), 1.86 (s, 3H), 1.82-1.50 (m, 5H), 1.63 (s, 3H), 1.14 (d, J = 6.0 Hz,3H), 1.13 (s, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 209.8, 169.9, 166.5, 162.6, 148.7, 122.3, 121.0, 117.3, 84.0, 81.5, 79.7, 63.0, 61.6, 56.2, 53.6,52.2, 50.8, 39.7, 38.7, 37.5, 35.5, 34.1,

33.2, 29.9, 24.5, 22.0, 21.6, 20.6, 17.9, 16.9, 16.1, 12.6; HRMS: $[M + Na]^+$ calcd for $C_{33}H_{45}NO_{10}Na$ 585.2226, found 585.2229; purity 95.78% ($MeOH/H_2O$ = 75/25, R_t = 3.686 min).

(3). To a solution of the **1** (26.3 mg) and 3-Chloroperbenzoic acid (8.7 mg) in anhydrous CH_2Cl_2, and the mixture was stirred at 0 °C for 12h. **3** was obtained as a white powder, 21.1mg, yield 78 %; m.p. 131-133 °C; **Crystal Data** for **3** (CCDC-2008080): orthorhombic, space group $P2_12_12_1$ (no. 19), a = 7.4020(2) Å, b = 13.4298(4) Å, c = 26.9766(9) Å, V = 2681.67(14) Å^3, Z = 4, T = 130.0 K, μ(CuKα) = 0.814 mm^{-1}, D_{calc} = 1.344 g/cm^3, 20906 reflections measured (6.552° ≤ 2Θ ≤ 149.01°), 5373 unique (R_{int} = 0.0737, R_{sigma} = 0.0636) which were used in all calculations. The final R_1 was 0.0468 (I > 2σ(I)) and wR_2 was 0.1108 (all data). ^{1}H NMR (500 MHz, $CDCl_3$) δ 6.94 (dd, J = 10.0 Hz, 6.0 Hz, 1H), 6.17 (d, J = 10.0 Hz, 1H), 4.99 (s, 1H), 4.49 (m, 1H), 3.80 (dd, J = 4.8 Hz, 1.8 Hz, 1H), 3.48 (s, 1H), 3.34 (s, 1H), 3.17 (brs, 1H), 2.57 (dt, J = 14.0 Hz, 3.0 Hz, 1H), 2.50 (m, 1H), 2.40 (t, J = 16.0 Hz, 1H), 2.11 (dd, J = 16.0 Hz, 3.5 Hz, 1H), 2.05 (s, 3H), 1.98 (dd, J = 10.5 Hz,2.5 Hz, 1H), 1.93 (s, 3H), 1.88 (s, 3H), 1.74-1.59 (m, 4H), 1.40 (s, 3H), 1.39 (m, 2H), 1.14 (s, 3H), 1.00 (d, J = 6.0 Hz, 3H); ^{13}C NMR (125 MHz, $CDCl_3$), δ 202.8, 169.5, 166.4, 149.3, 142.8, 131.3, 122.1, 81.1, 77.6, 76.8, 76.1, 69.4, 63.8, 63.0, 59.0, 47.6, 46.4, 38.9, 34.6, 33.8, 32.5, 31.5, 25.8, 21.3, 21.1, 20.6, 17.8, 15.7, 13.4, 12.6; HRMS: $[M + Na]^+$ calcd for $C_{30}H_{38}O_9Na$ 565.2408, found 565.2411; purity 97.91% ($MeOH/H_2O$ = 60/40-100/0, R_t = 6.117 min).

(4). To a solution of the **1** (26.3 mg) and MnO_2 (17.2 mg) in anhydrous CH_2Cl_2, and the mixture was stirred at 25 °C for 8h. **4** was obtained as a faint yellow powder, 21.2 mg, yield 81 %; m.p. 249-251 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 6.87 (q, 10.0 Hz, 2H), 5.73 (d, J = 2.8 Hz 1H), 5.22 (d, J = 2.8 Hz 1H), 4.24 (m, 1H), 3.57 (d, J = 2.5 Hz, 1H), 2.63 (dt, J = 14.0 Hz, 3.0 Hz, 1H), 2.52 (d, J = 7.5 Hz, 1H), 2.40 -2.33 (m, 2H), 2.18 (dd, J = 16.0 Hz, 3.5 Hz, 1H), 1.99 (s, 3H), 1.94 (s,

3H), 1.87 (s, 3H), 1.80-1.65 (m, 7H), 1.40 (s, 3H), 1.15 (d, J = 7.0 Hz, 3H), 1.11 (s, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 202.4, 193.8, 166.2, 162.3, 148.5, 141.3, 139.6, 122.4, 121.4, 83.8, 81.2, 79.3, 64.2, 63.7, 52.2, 50.2, 38.4, 37.7, 35.5, 34.6, 33.0, 29.9, 24.7, 23.3, 21.6, 20.6, 19.4, 17.7, 16.2, 12.6; HRMS: $[M + NH_4]^+$ calcd for $C_{30}H_{40}NO_8$ 542.2751, found 542.2750; purity 98.20% ($MeOH/H_2O$ = 60/40-100/0, R_t = 8.344 min).

(5). To a solution of the **1** (26.3 mg), TBDMSCl (17.2 mg) and imidazole(1.0 mg) anhydrous CH_2Cl_2, and the mixture was stirred at 25 °C for 12h. **5** was obtained as a faint yellow powder, 13.1 mg, yield 41 %; m.p. 194-196 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.13 (dd, J = 10.0 Hz, 6.0 Hz, 1H), 6.88 (d, J = 10.5 Hz, 1H), 5.76 (d, J = 3.0 Hz, 1H), 5.18 (d, J = 3.0 Hz, 1H), 4.48 (m, 1H), 4.22 (m, 1H), 3.37 (brs, 1H), 2.59 (dt, J = 14.0 Hz, 3.0 Hz, 1H), 2.50 (m, 1H), 2.40 (t, J = 16.0 Hz, 1H), 2.17 (dd, J = 16.0 Hz, 3.5 Hz, 1H), 2.11 (s, 3H), 1.93 (s, 3H), 1.86 (s, 3H), 1.86 (m, 1H), 1.75 -1.59 (m, 5H), 1.60 (s, 3H), 1.41 (m, 2H), 1.40 (s, 3H), 1.12 (d, J =7.5 Hz, 3H), 1.06 (s, 3H), 0.93 (s, 9H), 0.17 (s, 3H), 0.13 (s, 3H); ^{13}C NMR (125 MHz, $CDCl_3$), δ 169.7, 166.2, 162.3 148.5, 136.1, 130.0, 122.4, 121.5, 84.2, 81.4, 79.3, 79.0, 61.9, 60.1, 53.6, 52.3, 51.1, 39.2, 38.6, 37.3, 35.5, 34.4, 33.0, 32.1, 29.9, 27.4, 25.9, 24.7, 22.8, 21.7, 21.4, 20.6, 18.2, 17.7, 16.0, 14.3, 13.0; HRMS: $[M + Na]^+$ calcd for $C_{36}H_{52}O_8SiNa$ 663.3324, found 663.3326; purity 97.52% ($MeOH/H_2O$ = 60/40-100/0, R_t = 14.313 min).

(6). To a solution of the **1** (26.3 mg) in anhydrous pyridine (1.0 mL)was added *N,N'*-Carbonyldiimidazole (8.1 mg), and the mixture was stirred at 25 °C for 8h, Then add 0.5mL methanol, **6** was obtained as a faint yellow powder, 21.0 mg, yield 72 %; m.p. 118-120 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.05 (dd, J = 10.0 Hz, 6.0 Hz, 1H), 6.28 (d, J = 10.5 Hz, 1H), 5.69 (d, J = 3.0 Hz, 1H), 5.22 (d, J = 3.0 Hz, 1H), 4.65 (d, J = 6.5 Hz, 1H), 4.22 (m, 1H), 3.77 (s, 3H), 3.37 (brs, 1H), 2.59 (dt, J = 14.0 Hz, 3.0 Hz, 1H), 2.50 (m, 1H), 2.40 (t, J = 16.0 Hz, 1H), 2.17

(dd, J = 16.0 Hz, 3.5 Hz, 1H), 1.94 (s, 3H), 1.93 (s, 3H), 1.86 (s, 3H), 1.83 (m, 1H), 1.75 -1.59 (m, 5H), 1.60 (s, 3H), 1.41 (m, 2H), 1.39 (s, 3H), 1.11 (d, J =7.5 Hz, 3H), 1.08 (s, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 201.3, 169.7, 166.2, 162.5, 155.2,148.5, 139.7, 134.0, 122.4, 121.1, 83.7, 81.6, 79.4, 75.0, 61.4, 60.4, 55.3, 52.2, 48.0, 39.6, 37.4, 35.5, 34.6, 33.0, 24.6, 21.5, 21.2, 20.6, 17.7, 16.0, 15.9, 12.6; HRMS: $[M + NH_4]^+$ calcd for $C_{32}H_{44}NO_{10}$ 602.2960, found 602.2965; purity 98.20% ($MeOH/H_2O$ = 75/25, R_t = 4.337 min).

(7a). To a solution of the **1** (26.3 mg) in anhydrous pyridine (1.0 mL) was added Ac_2O (0.5 mL), and the mixture was stirred at 25 °C for 12h. **7a** was obtained as a faint yellow powder, 24.2 mg, yield 86 %; m.p. 126-128 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.07 (dd, J = 10.0 Hz, 6.0 Hz, 1H), 6.24 (d, J = 10.5 Hz, 1H), 5.69 (d, J = 3 Hz, 1H), 5.22 (d, J = 3 Hz, 1H), 4.70 (d, J = 6.5 Hz, 1H), 4.23 (m, 1H), 3.34 (brs, 1H), 2.57 (dt, J = 14.0 Hz, 3.0 Hz, 1H), 2.50 (m, 1H), 2.40 (t, J = 16.0 Hz, 1H), 2.17 (dd, J = 16.0 Hz, 3.5 Hz, 1H), 2.06 (s, 3H), 1.97 (m, 1H), 1.94 (s, 3H), 1.93 (s, 3H), 1.86 (s, 3H), 1.83 (m, 1H), 1.75 -1.59 (m, 5H), 1.60 (s, 3H), 1.41 (m, 2H), 1.40 (s, 3H), 1.12 (d, J =7.5 Hz, 3H), 1.09 (s, 3H); ^{13}C NMR (125MHz, $CDCl_3$), δ 201.5, 170.2, 169.7, 166.2, 162.5, 148.5, 140.7, 133.3, 122.4, 121.1, 83.7, 81.6, 79.4, 72.1, 61.1, 60.3, 52.2, 48.1, 39.7, 37.4, 35.5, 34.6, 33.0, 24.6, 21.5, 21.0, 20.6, 17.7, 16.0, 15.7, 12.6; HRMS: $[M + NH_4]^+$ calcd for $C_{32}H_{44}NO_9$ 586.3011, found 586.3013; purity 99.34% ($MeOH/H_2O$ = 60/40-100/0, R_t = 9.679 min).

(7b). To a solution of the **1** (26.3 mg) in anhydrous pyridine (1.0 mL) was added propionic anhydride (0.5 mL), and the mixture was stirred at 25 °C for 16h. **7b** was obtained as a faint yellow powder, 22.1 mg, yield 76 %; m.p. 105-107 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.07 (dd, J = 10.0 Hz, 6.0 Hz, 1H) , 6.24 (d, J = 10.5 Hz, 1H), 5.68 (d, J = 2.5 Hz, 1H), 5.22 (d, J = 2.5 Hz, 1H), 4.71 (d, J = 5.5 Hz, 1H), 4.23 (m, 1H), 3.34 (brs, 1H), 2.57 (dt, J = 14.0 Hz, 3.0 Hz,1H), 2.50 (m, 1H), 2.34 (m, 3H), 2.17 (dd, J = 16.0 Hz, 3.5 Hz, 1H), 2.04 (s, 3H), 1.94 (s, 3H), 1.93 (s, 3H), 1.85

(s, 3H), 1.84 -1.67 (m, 6H), 1.40 (s, 3H), 1.12- 1.10 (m, 5H), 1.08 (s, 3H); ^{13}C NMR (125 MHz, $CDCl_3$), δ 201.5, 173.6, 169.7, 166.3, 162.4, 148.4, 140.9, 133.2, 122.3, 121.1, 83.7, 81.6, 79.3, 71.9, 61.0, 52.2, 48.0, 39.6, 35.4, 34.6, 33.0, 27.5, 24.5, 21.5, 21.0, 20.6, 17.7, 16.0, 15.6, 12.6; HRMS: $[M + NH_4]^+$ calcd for $C_{33}H_{46}NO_9$ 600.3167, found 600.3169; purity 96.86% ($MeOH/H_2O$ = 60/40-100/0, R_t = 11.048 min).

(8a). To a solution of the crylic acid (7.2 mg), DMAP (4 mg) and EDCI (200 mg) in anhydrous CH_2Cl_2, add 0.5 mL TEA, and the mixture was stirred at 0 °C for 1h, Then add **1** (26.3 mg), and the mixture was stirred at 25 °C for 24h. **8a** was obtained as a yellow powder, 22.9 mg, yield 71 %; m.p. 99-101 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.49 (s, 1H), 7.38 (d, J = 13.0 Hz, 1H), 7.13 (dd, J = 10.0 Hz, 6.0 Hz, 1H), 6.63 (d, J = 3.0 Hz, 1H), 6.47 (q , J = 1.5 Hz, 1H), 6.27 (d , J = 3.5 Hz, 1H), 6.25 (d , J = 1.5 Hz, 1H) 5.69 (d, J = 2.5 Hz, 1H), 5.23 (d, J = 2.5 Hz, 1H), 4.82 (d, J = 10.0 Hz, 1H), 4.23 (m, 1H), 3.38 (brs, 1H), 2.57 (dt, J = 14.0 Hz, 3.0 Hz, 1H), 2.50 (m, 1H), 2.40 (t, J = 16.0 Hz, 1H), 2.18 (dd, J = 16.0 Hz, 3.5 Hz, 1H), 1.95 (s, 3H), 1.93(s, 3H), 1.86 (s, 3H), 1.82 (m, 1H), 1.74 -1.59 (m, 5H), 1.42 (s, 3H), 1.41 (m, 2H), 1.12 (d, J = 7.5 Hz, 3H), 1.09 (s, 3H); ^{13}C NMR (125 MHz, $CDCl_3$), δ 201.4, 169.7, 166.2, 162.5, 148.5, 140.6, 133.5, 132.1, 127.8, 122.4, 121.1, 83.8, 81.7, 79.4, 72.2, 61.1, 60.4, 52.2, 48.1, 39.4, 37.4, 35.5, 34.6, 33.0, 29.9, 24.6, 21.5, 21.0, 20.6, 17.7, 16.0, 15.7, 12.6; HRMS: $[M + NH_4]^+$ calcd for $C_{33}H_{44}NO_9$ 598.3011, found 598.3010; purity 98.36% ($MeOH/H_2O$ = 75/25, R_t = 7.101 min).

(8b). To a solution of the crotonic acid (8.6 mg), DMAP (4mg) and EDCI (200 mg) in anhydrous CH_2Cl_2, add 0.5 mL TEA, and the mixture was stirred at 0 °C for 1h, Then add **1**(26.3 mg), and the mixture was stirred at 25 °C for 24h. **8b** was obtained as a faint yellow powder, 17.8 mg, yield 60 %; m.p. 116-118 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.11 (dd, J = 10.0 Hz, 6.0 Hz, 1H), 6.95 (q, J = 6.5 Hz, 1H), 6.24 (d, J = 10.0 Hz, 1H), 5.81 (dd, J = 13.0 Hz, 1.5 Hz 1H), 5.69 (d, J = 2.5

Hz, 1H), 5.22 (d, *J* = 2.5 Hz, 1H), 4.76 (d, *J* = 5.0 Hz, 1H), 4.23 (m, 1H), 3.36 (brs, 1H), 2.57 (dt, *J* = 14.0 Hz, 3.0 Hz, 1H), 2.50 (m, 1H), 2.40 (t, *J* = 16.0 Hz, 1H), 2.17 (dd, *J* = 16.0 Hz, 3.5 Hz, 1H), 1.94 (s, 3H), 1.93(s, 3H), 1.88 (dd, *J* = 6.0 Hz, 1.5 Hz 3H), 1.86 (s, 3H), 1.82 (m, 1H), 1.74 -1.59 (m, 5H), 1.42 (s, 3H), 1.41 (m, 2H), 1.12 (d, *J* = 7.5 Hz, 3H), 1.09 (s, 3H); ^{13}C NMR (125 MHz, $CDCl_3$), *δ* 201.5, 169.7, 166.2, 165.5, 162.5, 148.5, 146.4, 141.0, 133.2, 122.4, 121.9, 121.1, 83.7, 81.7, 79.3, 71.8, 61.0, 60.4, 52.2, 48.1, 39.7, 37.3, 35.4, 34.6, 33.0, 24.6, 21.5, 21.0, 20.6, 18.3, 17.6, 16.0, 15.7, 12.6; HRMS: $[M + NH_4]^+$ calcd for $C_{34}H_{46}NO_9$ 612.3167, found 612.3164; purity 95.25% (MeOH/H_2O = 75/25, R_t = 7.645 min).

(8c). To a solution of the 3-methylbut-2-enoic acid (10.0 mg), DMAP (4 mg) and EDCI (200 mg) in anhydrous CH_2Cl_2, add 0.5 mL TEA, and the mixture was stirred at 0 °C for 1h, Then add **1** (26.3 mg), and the mixture was stirred at 25 °C for 24h. **8c** was obtained as a faint yellow powder, 20.7 mg, yield 68 %; m.p. 108-110 °C; ^{1}H NMR (500 MHz, $CDCl_3$) *δ* 7.07 (dd, *J* = 10.0 Hz, 6.0 Hz, 1H), 6.24 (d, *J* = 10.0 Hz, 1H), 5.68 (d, *J* = 2.5 Hz, 1H), 5.22 (d, *J* = 2.5 Hz, 1H), 4.90 (t, *J* = 1.5 Hz, 1H), 4.83 (s, 1H), 4.72 (d, *J* = 10.0 Hz, 1H), 4.23 (m, 1H), 3.35 (brs, 1H), 3.04 (s, 2H), 2.57 (dt, *J* = 14.0 Hz, 3.0 Hz, 1H), 2.50 (m, 1H), 2.40 (t, *J* = 16.0 Hz, 1H) , 2.17 (dd, *J* = 16.0 Hz, 3.5 Hz, 1H), 1.94 (s, 3H), 1.93 (s, 3H), 1.86 (s, 3H), 1.83 (m, 1H), 1.76 (s, 3H), 1.83 -1.59 (m, 5H), 1.40 (m, 2H), 1.39 (s, 3H), 1.12 (d, *J* = 7.5 Hz, 3H), 1.09 (s, 3H); ^{13}C NMR (125 MHz, $CDCl_3$), *δ* 201.5, 170.6, 169.7, 166.2, 162.5, 148.6, 140.5, 137.9, 133.4, 122.4, 121.1, 115.4, 83.7, 81.6, 79.4, 72.2, 61.1, 60.3, 52.2, 48.1, 43.4, 39.7, 37.4, 35.5, 34.6, 33.0, 24.6, 22.5, 21.5, 21.0, 20.6, 17.7, 16.0, 15.6, 12.6; HRMS: $[M + NH_4]^+$ calcd for $C_{35}H_{48}NO_9$ 626.3324, found 626.3325; purity 96.96% (MeOH/H_2O = 75/25, R_t = 10.075 min).

(8d). To a solution of the sorbic acid (11.2 mg), DMAP (4 mg) and EDCI (200 mg) in anhydrous CH_2Cl_2, add 0.5 mL TEA, and the mixture was stirred at 0 °C for 1h, Then add **1** (26.3 mg), and

the mixture was stirred at 25 °C for 24h. **8d** was obtained as a faint yellow powder, 22.9 mg, yield 74 %; m.p. 91-93 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.20 (dd, J = 10.0 Hz, 6.0 Hz, 1H), 7.11 (q, J = 6.5 Hz, 1H), 6.24 (d, J = 10.0 Hz, 1H), 6.16 (m, 1H), 5.73 (d, J = 15.0 Hz, 1H), 5.69 (d, J = 2.5 Hz, 1H), 5.22 (d, J = 2.5 Hz, 1H), 4.77 (d, J = 5.0 Hz, 1H), 4.23 (m, 1H), 3.36 (brs, 1H), 2.57 (dt, J = 14.0 Hz, 3.0 Hz, 1H), 2.50 (m, 1H), 2.40 (t, J = 16.0 Hz, 1H), 2.17 (dd, J = 16.0 Hz, 3.5 Hz, 1H), 1.94 (s, 3H), 1.93(s, 3H), 1.86 (s, 3H), 1.85 (s, 3H), 1.82 (m, 1H), 1.74 -1.59 (m, 5H), 1.43 (s, 3H), 1.42 (m, 2H), 1.12 (d, J = 7.5 Hz, 3H), 1.09 (s, 3H); ^{13}C NMR (125 MHz, $CDCl_3$), δ 201.6, 169.7, 166.4, 166.2, 162.4, 148.5, 146.5, 141.1, 140.7, 133.2, 129.8, 122.4, 121.1, 117.9, 83.7, 81.7 79.3, 71.8, 61.0, 53.6, 52.2, 48.1, 39.7, 37.3, 35.4, 34.6, 32.9, 24.6, 21.5, 21.0, 20.6, 18.9, 17.6, 16.0, 15.7, 12.6; HRMS: $[M + NH_4]^+$ calcd for $C_{36}H_{48}NO_9$, 638.3324; found 638.3327; purity 96.13% (MeOH/H_2O = 60/40-100/0, R_t = 13.488 min).

(8e). To a solution of the monoethyl fumarate (14.4 mg), DMAP (4 mg) and EDCI (200 mg) in anhydrous CH_2Cl_2, add 0.5 mL TEA, and the mixture was stirred at 0 °C for 1h, Then add **1** (26.3 mg), and the mixture was stirred at 25 °C for 24h. **8e** was obtained as a yellow powder, 25.8 mg, yield 79 %; m.p. 93-95 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.07 (dd, J = 10.0 Hz, 6.0 Hz, 1H), 6.81 (s, 2H), 6.28 (d, J = 10.0 Hz, 1H), 5.69 (d, J = 2.5 Hz, 1H), 5.22 (d, J = 2.5 Hz, 1H), 4.83 (d, J = 10.0 Hz, 1H), 4.27 (q, J = 6.0 Hz, 1H), 4.23 (m, 1H), 3.38 (brs, 1H), 3.04 (s, 2H) , 2.57 (dt, J = 14.0 Hz, 3.0 Hz, 1H), 2.50 (m, 1H), 2.40 (t, J = 16.0 Hz, 1H), 2.17 (dd, J = 16.0 Hz, 3.5 Hz, 1H), 1.94 (s, 3H), 1.93(s, 3H), 1.86 (s, 3H), 1.83 -1.67 (m, 6H), 1.43 (s, 3H), 1.40 (m, 2H), 1.32 (t, J = 6.0 Hz, 3H), 1.12 (d, J = 7.5 Hz, 3H), 1.09 (s, 3H); ^{13}C NMR (125 MHz, $CDCl_3$), δ 201.2, 169.7, 166.2, 164.8, 164.2, 162.5, 148.6, 139.8, 135.0, 133.9, 132.5, 122.4, 121.0, 83.7, 81.6, 79.4, 72.9, 61.7, 61.3, 60.2, 52.2, 48.0, 39.6, 37.4, 35.5, 34.5, 33.1, 24.5, 21.5, 21.0, 20.6, 17.8, 16.0,

15.9, 14.3, 12.6; HRMS: $[M + Na]^+$ calcd for $C_{36}H_{44}O_{11}Na$ 675.2776, found 675.2778. Purity 95.43% ($MeOH/H_2O$ = 75/25, R_t = 7.769 min).

(8f). To a solution of the 3-Ethoxyacrylic Acid (11.6 mg), DMAP (4 mg) and EDCI (200 mg) in anhydrous CH_2Cl_2, add 0.5 mL TEA, and the mixture was stirred at 0 °C for 1h, Then add **1** (26.3 mg), and the mixture was stirred at 25 °C for 24h. **8f** was obtained as a yellow powder, 21.5 mg, yield 69 %; m.p. 124-126 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.55 (d, *J* = 11.0 Hz, 1H), 7.11 (dd, *J* = 10.0 Hz, 6.0 Hz, 1H), 6.23 (d, *J* = 10.5 Hz, 1H), 5.69 (d, *J* = 3 Hz, 1H), 5.22 (d, *J* = 3.0 Hz, 1H), 5.13 (d, *J* = 11.0 Hz, 1H), 4.76 (d, *J* = 6.0 Hz, 1H), 4.23 (m, 1H), 3.90 (q, *J* = 6.0 Hz, 2H), 3.35 (brs, 1H), 2.57 (dt, *J* = 14.0 Hz, 3.0 Hz, 1H), 2.50 (m, 1H), 2.40 (t, *J* = 16.0 Hz, 1H), 2.17 (dd, *J* = 16.0 Hz, 3.5 Hz, 1H), 2.04 (s, 4H), 1.94 (s, 3H), 1.93 (s, 3H), 1.86 (s, 3H), 1.83 (m, 1H), 1.75 -1.59 (m, 5H), 1.41 (s, 3H), 1.41 (m, 1H), 1.38 (m, 1H), 1.33 (t, *J* = 6.0 Hz, 3H), 1.12 (d, *J* =7.5 Hz, 3H), 1.08 (s, 3H); ^{13}C NMR (125 MHz, $CDCl_3$), δ 201.6, 169.7, 167.0, 166.2, 163.5, 162.4, 148.6, 141.4, 133.0, 122.4, 121.1, 95.5, 83.7, 81.6, 79.3, 71.3, 67.0, 61.0, 60.5, 53.6, 52.2, 48.1, 39.7, 37.4, 35.4, 34.6, 33.0, 24.6, 21.5, 21.0, 20.6, 17.6, 16.0, 15.7, 14.5, 12.6; HRMS: $[M + Na]^+$ calcd for $C_{35}H_{44}O_{10}Na$ 647.2827, found 647.2828; purity 95.08% ($MeOH/H_2O$ = 75/25, R_t = 7.960 min).

(8g). To a solution of the 3- (2-Furyl) acrylic acid (13.7 mg), DMAP (4 mg) and EDCI (200 mg) in anhydrous CH_2Cl_2, add 0.5 mL TEA, and the mixture was stirred at 0 °C for 1h, Then add **1** (26.3 mg), and the mixture was stirred at 25 °C for 24h. **8g** was obtained as a yellow powder, 22.9 mg, yield 71 %; m.p. 120-122 °C; ^{1}H NMR (500 MHz, $CDCl_3$) *δ* 7.49 (s, 1H), 7.38 (d, *J* = 13.0 Hz, 1H), 7.13 (dd, *J* = 10.0 Hz, 6.0 Hz, 1H), 6.63 (d, *J* = 3.25 Hz, 1H), 6.47 (q, 1H), 6.27 (d, *J* = 3.25 Hz, 1H), 6.25 (d, *J* = 1.75 Hz, 1H), 5.69 (d, *J* = 2.5 Hz, 1H), 5.23 (d, *J* = 2.5 Hz, 1H), 4.82 (d, *J* = 5.0 Hz, 1H), 4.23 (m, 1H), 3.38 (brs, 1H), 2.57 (dt, *J* = 14.0 Hz, 3.0 Hz, 1H), 2.50 (m, 1H),

2.40 (t, J = 16.0 Hz, 1H), 2.17 (dd, J = 16.0 Hz, 3.5 Hz, 1H), 1.95 (s, 3H), 1.93(s, 3H), 1.86 (s, 3H), 1.83 (m, 1H), 1.74 -1.59 (m, 5H), 1.46 (s, 3H), 1.40 (m, 2H), 1.12 (d, J = 7.5 Hz, 3H), 1.09 (s, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 201.6, 169.7, 166.3, 166.2, 162.4, 150.8, 148.6, 145.2, 140.9, 133.4, 132.2, 122.3, 121.0, 115.7, 114.7, 112.6, 83.7, 81.6, 79.3, 72.0, 61.1, 60.5, 52.2, 48.1, 39.7, 37.4, 35.5, 34.6, 33.0, 24.6, 21.5, 21.0, 20.6, 17.7, 16.0, 15.8, 12.6; HRMS: $[M+NH_4]^+$ calcd for $C_{37}H_{46}NO_{10}$, 664.3116, found 664.3121; purity 97.12% (MeOH/H_2O = 75/25, R_t = 10.616 min).

(8h). To a solution of the 3-(2-Thienyl)Acrylic Acid (15.4 mg), DMAP (4 mg) and EDCI (200 mg) in anhydrous CH_2Cl_2, add 0.5 mL TEA, and the mixture was stirred at 0 °C for 1h, Then add **1** (26.3 mg), and the mixture was stirred at 25 °C for 24h. **8h** was obtained as a yellow powder, 19.6 mg, yield 59 %; m.p. 83-85 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.75(d, J = 13.5 Hz, 1H), 7.40 (d, J = 4.5 Hz, 1H), 7.13 (dd, J = 10.0 Hz, 6.0 Hz, 1H), 7.06 (dd, J = 13.5 Hz, 4.5 Hz, 1H), 6.27 (d, J = 8.0 Hz, 1H), 6.17 (d, J = 8.0 Hz, 1H), 6.27 (d, J = 13.0 Hz, 1H), 6.25 (d, J = 1.75 Hz, 1H), 5.69 (d, J = 2.5 Hz, 1H), 5.23 (d, J = 2.5 Hz, 1H), 4.83 (d, J = 5.0 Hz, 1H), 4.23 (m, 1H), 3.39 (brs, 1H), 2.57 (dt, J = 14.0 Hz, 3.0 Hz, 1H), 2.50 (m, 1H), 2.40 (t, J = 16.0 Hz, 1H), 2.17 (dd, J = 16.0 Hz, 3.5 Hz, 1H), 1.95 (s, 3H), 1.93 (s, 3H), 1.86 (s, 3H), 1.83 (m, 1H), 1.74 -1.59 (m, 5H), 1.47 (s, 3H), 1.40 (m, 2H), 1.12 (d, J = 7.5 Hz, 3H), 1.10 (s, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 201.5, 169.7, 166.2, 166.0, 162.5, 148.6, 140.9, 139.4, 138.5, 133.4, 131.7, 129.2, 128.3, 122.4, 121.1, 115.7, 83.7, 81.7, 79.3, 72.1, 61.1, 60.4, 53.6, 52.2, 48.1, 39.7, 37.4, 35.5, 34.6, 33.0, 24.6, 21.6, 21.0, 20.6, 17.7, 16.0, 15.9, 12.6; HRMS: $[M+NH_4]^+$ calcd for $C_{37}H_{46}NO_{10}SNa$ 680.2888, found 580.2891; purity 99.39% (MeOH/H_2O = 75/25, R_t = 10.115 min).

(8i). To a solution of the cinnamic acid (14.8 mg), DMAP (4 mg) and EDCI (200 mg) in anhydrous CH_2Cl_2, add 0.5 mL TEA, and the mixture was stirred at 0 °C for 1h, Then add **1** (26.3 mg), and

the mixture was stirred at 25 °C for 24h. **8i** was obtained as a yellow powder, 12.4 mg, yield 37 %; m.p. 104-106 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.65 (d, J = 13.5 Hz, 1H), 7.51 (m, 2H), 7.38 (m, 2H), 7.15 (dd, J = 10.0 Hz, 6.0 Hz, 1H), 6.39 (d, J = 13.5 Hz, 1H), 6.28 (d, J = 8.25 Hz, 1H), 6.27 (d, J = 13.0 Hz, 1H), 5.69 (d, J = 2.5 Hz, 1H), 5.23 (d, J = 2.5 Hz, 1H), 4.84 (d, J = 5.5 Hz, 1H), 4.23 (m, 1H), 3.40 (brs, 1H), 2.57 (dt, J = 14.0 Hz, 3.0 Hz, 1H), 2.50 (m, 1H), 2.40 (t, J = 16.0 Hz, 1H), 2.17 (dd, J = 16.0 Hz, 3.5 Hz, 1H), 2.04 (s, 1H), 1.95 (s, 3H), 1.93 (s, 3H), 1.86 (s, 3H), 1.83 (m, 1H), 1.74 -1.59 (m, 5H), 1.48 (s, 3H), 1.40 (m, 2H), 1.12 (d, J = 7.5 Hz, 3H), 1.10 (s, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 201.5, 169.7, 166.2, 166.1, 162.5, 148.6, 140.9, 134.2, 133.4, 131.7, 130.8, 129.1, 128.4, 122.4, 121.1, 117.1, 83.7, 81.6, 79.3, 72.1, 61.1, 60.4, 53.6, 52.2, 48.1, 39.7, 37.4, 35.4, 34.6, 33.0, 24.6, 21.5, 21.0, 20.6, 17.7, 16.0, 15.8, 12.6; HRMS: $[M + Na]^+$ calcd for $C_{39}H_{44}O_9Na$ 679.2878, found 679.2880; purity 97.71% (MeOH/H_2O = 75/25, R_t = 13.904 min).

(8j). To a solution of the 4-Fluorocinnamic acid (16.6 mg), DMAP (4 mg) and EDCI (200 mg) in anhydrous CH_2Cl_2, add 0.5 mL TEA, and the mixture was stirred at 0 °C for 1h, Then add **1** (26.3 mg), and the mixture was stirred at 25 °C for 24h. **8j** was obtained as a yellow powder, 17.9 mg, yield 53.0 %; m.p. 127-129 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.61 (d, J = 13.5 Hz, 1H), 7.50 (m, 2H), 7.16 (dd, J = 10.0 Hz, 6.0 Hz, 1H), 7.08 (m, 2H), 6.29 (m, 2H), 5.69 (d, J = 2.5 Hz, 1H), 5.23 (d, J = 2.5 Hz, 1H), 4.84 (d, J = 5.5 Hz, 1H), 4.23 (m, 1H), 3.40 (brs, 1H), 2.57 (dt, J = 14.0 Hz, 3.0 Hz, 1H), 2.50 (m, 1H), 2.40 (t, J = 16.0 Hz, 1H), 2.17 (dd, J = 16.0 Hz, 3.5 Hz, 1H), 2.04 (s, 1H), 1.95 (s, 3H), 1.93 (s, 3H), 1.86 (s, 3H), 1.83 (m, 1H), 1.74 -1.59 (m, 5H), 1.47 (s, 3H), 1.40 (m, 2H), 1.12 (d, J = 7.5 Hz, 3H), 1.10 (s, 3H); ^{13}C NMR (125MHz, $CDCl_3$) δ 201.5, 169.7, 166.2, 166.0, 162.5, 148.5, 144.9, 140.8, 133.4, 130.3, 130.3, 122.4, 121.1, 116.9, 116.3, 116.2, 83.7, 81.7, 79.4, 72.2, 61.1, 60.5, 52.2, 48.1, 39.7, 37.4, 35.5, 34.6, 33.1, 24.6, 21.5, 21.0, 20.6, 17.7,

16.0, 15.8, 12.6; HRMS: $[M + Na]^+$ calcd for $C_{39}H_{43}FO_9Na$ 697.2783, found 697.2781; purity 95.36% ($MeOH/H_2O$ = 75/25, R_t = 13.000 min).

(8k). To a solution of the 3- (Trifluoromethyl)cinnamic acid (21.6 mg), DMAP (4 mg) and EDCI (200 mg) in anhydrous CH_2Cl_2, add 0.5 mL TEA, and the mixture was stirred at 0 °C for 1h, Then add **1** (26.3 mg), and the mixture was stirred at 25 °C for 24h. **8k** was obtained as a yellow powder, 24.3 mg, yield 67 %; m.p. 129-131 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.74 (s, 1H), 7.67 (m, 3H), 7.52 (m, 1H), 7.14 (dd, J = 10.0 Hz, 6.0 Hz, 1H), 6.45 (d, J = 13.5 Hz, 1H), 6.29 (d, J = 10.0 Hz, 1H), 5.69 (d, J = 2.5 Hz, 1H), 5.24 (d, J = 2.5 Hz, 1H), 4.87 (d, J = 5.5 Hz, 1H), 4.23 (m, 1H), 3.40 (brs, 1H), 2.57 (dt, J = 14.0 Hz, 3.0 Hz, 1H), 2.50 (m, 1H), 2.40 (t, J = 16.0 Hz, 1H), 2.17 (dd, J = 16.0 Hz, 3.5 Hz, 1H), 2.04 (s, 1H), 1.95 (s, 3H), 1.93 (s, 3H), 1.86 (s, 3H), 1.83 (m, 1H), 1.74 -1.59 (m, 5H), 1.48 (s, 3H), 1.40 (m, 2H), 1.12 (d, J = 7.5 Hz, 3H), 1.10 (s, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 201.4, 169.7, 166.2, 165.6, 162.5, 148.5, 144.3, 140.5, 135.0, 133.6, 131.3, 129.7, 124.9, 122.4, 121.1, 119.2, 100.1, 83.7, 81.6, 79.4, 72.4, 61.2, 60.4, 53.6, 52.2, 48.1, 39.7, 37.4, 35.5, 34.6, 33.1, 29.9, 24.6, 21.5, 21.0, 20.6, 17.7, 16.0, 15.9, 12.6; HRMS: $[M + Na]^+$ calcd for $C_{40}H_{43}F_3O_9Na$ 747.2751, found 747.2750; purity 96.89% ($MeOH/H_2O$ = 75/25, R_t = 12.144 min).

(8l). To a solution of the methacrylic acid (8.6 mg), DMAP (4 mg) and EDCI (200 mg) in anhydrous CH_2Cl_2, add 0.5 mL TEA, and the mixture was stirred at 0 °C for 1h, Then add **1** (26.3 mg), and the mixture was stirred at 25 °C for 24h. **8l** was obtained as a white powder, 14.0 mg, yield 47 %; m.p. 105-107 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.12 (dd, J = 10.0 Hz, 6.0 Hz,1H), 6.26 (d, J = 10.0 Hz, 1H), 6.08 (s, 1H), 5.69 (d, J = 2.5 Hz, 1H), 5.60 (s, 1H), 5.23 (d, J = 2.5 Hz, 1H), 4.77 (d, J = 10.0 Hz, 1H), 4.23 (m, 1H), 3.37 (brs, 1H), 2.57 (dt, J = 14.0 Hz, 3.0 Hz, 1H), 2.50 (m, 1H), 2.40 (t, J = 16.0 Hz, 1H), 2.17 (dd, J = 16.0 Hz, 3.5 Hz, 1H), 1.94 (s, 3H), 1.93 (

s, 3H), 1.92 (s, 3H), 1.86 (s, 3H), 1.83 (m, 1H), 1.74 -1.59 (m, 5H), 1.43 (s, 3H), 1.40 (m, 2H), 1.12 (d, J = 7.5 Hz, 3H), 1.09 (s, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 201.4,169.7, 166.6, 166.2, 162.5, 148.5, 140.8, 135.6, 133.4, 127.0, 122.4, 121.1, 83.7, 81.7, 79.3, 72.3, 61.0, 60.41, 53.6, 52.2, 48.0, 39.7, 37.4, 35.4, 34.6, 33.0, 29.9, 24.6, 21.5, 21.0, 20.6, 18.5, 17.7, 16.0, 15.6, 12.6; HRMS: $[M + Na]^+$ calcd for $C_{38}H_{47}NO_{10}Na$ 617.2721, found 617.2725; purity 95.77% ($MeOH/H_2O$ = 60/40-100/0, R_t = 11.96 min).

(9a). To a solution of the **1** (26.3 mg), DMAP (4 mg) in anhydrous CH_2Cl_2 was added dimethylcarbamyl chloride (0.2 mL) and TEA (0.5 mL), and the mixture was stirred at 0 °C for 12 h. **9a** was obtained as a white powder, 22.4 mg, yield 75 %; m.p. 109-111 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.07 (dd, J = 10.0 Hz, 6.0 Hz, 1H), 6.23 (d, J = 10.0 Hz, 1H), 5.68 (d, J = 2.5 Hz, 1H), 5.22 (d, J = 2.5 Hz, 1H), 4.64 (d, J = 6.0 Hz, 1H), 4.23 (m, 1H), 3.35 (brs, 1H), 2.89 (s, 3H), 2.87 (s, 3H), 2.57 (dt, J = 14.0 Hz, 3.5 Hz, 1H), 2.50 (m, 1H), 2.40 (t, J = 16.0 Hz, 1H), 2.17 (dd, J = 16.0 Hz, 3.5 Hz, 1H), 1.94 (s, 3H), 1.93 (s, 3H), 1.86 (s, 3H), 1.83 (m, 1H), 1.75 -1.59 (m, 5H), 1.60 (s, 3H), 1.41 (m, 2H), 1.40 (s, 3H), 1.12 (d, J =7.5 Hz, 3H), 1.09 (s, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 201.7, 169.7, 166.2, 162.4, 155.6, 148.5, 142.1, 132.9, 122.4, 121.2, 83.7, 81.7, 79.3, 72.6, 61.0, 60.5, 52.2, 48.0, 39.6, 37.4, 36.8, 36.3, 33.0, 24.6, 21.5, 21.0, 20.6, 17.6, 16.0, 15.5, 12.6; HRMS: $[M + Na]^+$ calcd for $C_{33}H_{43}NO_9Na$ 620.2830, found 620.2830; purity 95.26% ($MeOH/H_2O$ = 60/40-100/0, R_t = 8.098 min).

(9b). To a solution of the **1** (26.3 mg), DMAP (4 mg) in anhydrous CH_2Cl_2 was added DL-2-Methylbutyryl chloride (0.2 mL) and TEA (0.5 mL), and the mixture was stirred at 0 °C for 12 h. **9b** was obtained as a white powder, 21.4 mg, yield 70 %; m.p. 111-113 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.12 (dd, J = 10.0 Hz, 6.0 Hz, 1H), 6.23 (d, J = 10.0 Hz, 1H), 5.68 (d, J = 2.5 Hz, 1H), 5.22 (d, J = 2.5 Hz, 1H), 4.62 (d, J = 6.0 Hz, 1H), 4.23 (m, 1H), 3.34 (brs, 1H), 2.84 (m, 4H), 2.57

(dt, J = 14.0 Hz, 3.5 Hz, 1H), 2.50 (m, 1H), 2.40 (t, J = 16.0 Hz, 1H), 2.17 (dd, J = 16.0 Hz, 3.5 Hz, 1H), 1.93 (s, 3H), 1.92 (s, 3H), 1.85 (s, 3H), 1.75 -1.59 (m, 5H), 1.38 (m, 6H), 1.10 (m, 12H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 201.7, 169.7, 166.4, 162.4, 155.3, 148.7, 142.6, 132.8, 130.0, 122.3, 121.0, 83.7, 81.6, 79.3, 72.5, 61.0, 60.5, 53.6, 52.2, 48.0, 39.6, 37.4, 36.8, 36.3, 34.6, 33.0, 29.8, 24.5, 21.5, 20.6, 17.7, 16.0, 15.3, 14.3, 12.6; HRMS: $[M + Na]^+$ calcd for $C_{34}H_{45}NO_9Na$ 634.2987, found 634.2988; purity 97.09% ($MeOH/H_2O$ = 60/40-100/0, R_t = 10.990 min).

(9c). To a solution of the **1** (26.3 mg), DMAP (4 mg) in anhydrous CH_2Cl_2 was added diethylcarbamyl chloride (0.2 mL) and TEA (0.5 mL), and the mixture was stirred at 0 °C for 12 h. **9c** was obtained as a white powder, 22.4 mg, yield 73 % ; m.p.: 224-225 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.15 (dd, J = 10.0 Hz, 6.0 Hz, 1H), 6.23 (d, J = 10.0 Hz, 1H), 5.68 (d, J = 2.5 Hz, 1H), 5.22 (d, J = 2.5 Hz, 1H), 4.63 (d, J = 6.0 Hz, 1H), 4.23 (m, 1H), 3.35 (brs, 1H), 3.04 (m, 4H), 2.57 (dt, J = 14.0 Hz, 3.5 Hz, 1H), 2.50 (m, 1H), 2.40 (t, J = 16.0 Hz, 1H), 2.17 (dd, J = 16.0 Hz, 3.5 Hz, 1H), 1.94 (s, 3H), 1.93 (s, 3H), 1.85 (s, 3H), 1.83 (m, 1H), 1.75 -1.59 (m, 5H), 1.48 (t, J =7.5 Hz, 6H), 1.41 (m, 2H), 1.38 (s, 3H), 1.11 (d, J =7.5 Hz, 3H), 1.08 (s, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 201.77, 169.69, 166.32, 162.42, 154.95, 148.62, 142.36, 132.75, 122.36, 121.05, 83.72, 81.66, 79.34, 72.47, 61.03, 60.54, 52.20, 48.00, 42.35, 39.66, 37.46, 35.52, 34.70, 33.08, 32.07, 29.84, 27.36, 24.60, 22.83, 21.47, 20.99, 20.58, 17.72, 16.00, 15.27, 14.25, 12.58, 11.37; HRMS: $[M + H]^+$ calcd for $C_{35}H_{48}NO_9$ 626.3324, found 626.3322; purity 95.66% ($MeOH/H_2O$ = 60/40-100/0, R_t = 11.005 min).

(9d). To a solution of the **1** (26.3 mg), DMAP (4 mg) in anhydrous CH_2Cl_2 was added 1-Pyrrolidinecarbonyl chloride (0.2 mL) and TEA (0.5 mL), and the mixture was stirred at 0 °C for 12 h. **9d** was obtained as a white powder, 16.2 mg, yield 52 %; m.p. 98-100 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.15 (dd, J = 10.0 Hz, 6.0 Hz, 1H), 6.23 (d, J = 10.0 Hz, 1H), 5.68 (d, J = 2.5 Hz,

1H), 5.22 (d, *J* = 2.5 Hz, 1H), 4.63 (d, *J* = 6.0 Hz, 1H), 4.23 (m, 1H), 3.38-3.23 (m, 5H), 2.57 (dt, *J* = 14.0 Hz, 3.5 Hz, 1H), 2.50 (m, 1H), 2.40 (t, *J* = 16.0 Hz, 1H), 2.17 (dd, *J* = 16.0 Hz, 3.5 Hz, 1H), 1.94 (s, 3H), 1.93 (s, 3H), 1.86 (s, 3H), 1.83 (m, 5H), 1.75 -1.59 (m, 5H), 1.41 (m, 2H), 1.40 (s, 3H), 1.12 (d, *J* =7.5 Hz, 3H), 1.09 (s, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 201.8, 169.7, 166.2, 162.5, 153.9, 148.5, 142.3, 132.8, 122.4, 121.1, 83.7, 81.7, 79.3, 72.3, 60.9, 60.6, 52.2, 48.0, 46.5, 46.2, 39.7, 37.4, 35.5, 34.7, 33.0, 32.1, 29.9, 25.8, 25.1, 24.6, 22.8, 21.5, 21.0, 20.6, 17.7, 16.0, 15.4, 14.3, 12.6; HRMS: $[M + Na]^+$ calcd for $C_{35}H_{45}NO_9Na$ 646.2987, found 646.2989; purity 95.24% ($MeOH/H_2O$ = 60/40-100/0, R_t = 12.44 min).

(9e). To a solution of the **1** (26.3 mg), DMAP (4 mg) in anhydrous CH_2Cl_2 was added piperidine-1-carbonyl chloride (0.2 mL) and TEA (0.5 mL), and the mixture was stirred at 0 °C for 12 h. **9e** was obtained as a white powder, 12.4 mg, yield 39 % ; m.p. 97-99 °C;^{1}H NMR (500 MHz, $CDCl_3$) δ 7.12 (dd, *J* = 10.0 Hz, 6.0 Hz, 1H), 6.23 (d, *J* = 10.0 Hz, 1H), 5.68 (d, *J* = 2.5 Hz, 1H), 5.22 (d, *J* = 2.5 Hz, 1H), 4.68 (d, *J* = 6.0 Hz, 1H), 4.23 (m, 1H), 3.39-3.35 (m, 5H), 2.57 (dt, *J* = 14.0 Hz, 3.5 Hz, 1H), 2.50 (m, 1H), 2.40 (t, *J* = 16.0 Hz, 1H), 2.17 (dd, *J* = 16.0 Hz, 3.5 Hz, 1H), 1.94 (s, 3H), 1.93 (s, 3H), 1.86 (s, 3H), 1.75 -1.59 (m, 11H), 1.41 (m, 2H), 1.38 (s, 3H), 1.12 (d, *J* =7.5 Hz, 3H), 1.08 (s, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 201.8, 169.7, 166.2, 162.4, 154.5, 148.5, 142.2, 132.8, 130.8, 122.4, 121.1, 83.7, 81.7, 79.3, 72.4, 61.6, 60.6, 52.2, 48.0, 45.3, 39.7, 37.5, 35.5, 34.7, 33.0, 29.9, 27.4, 24.6, 24.4, 22.8, 21.5, 21.1, 20.6, 17.7, 16.0, 15.6, 14.3, 12.6; HRMS: $[M + Na]^+$ calcd for $C_{36}H_{47}NO_9Na$ 660.3143, found 660.3145; purity 97.48% ($MeOH/H_2O$ = 60/40-100/0, R_t = 12.493 min).

(9f). To a solution of the **1** (26.3 mg), DMAP (4 mg) in anhydrous CH_2Cl_2 was added 4-Morpholinecarbonyl chloride (0.2 mL) and TEA (0.5 mL), and the mixture was stirred at 0 °C for 12 h. **9f** was obtained as a white powder, 13.4 mg, yield 42 %.; m.p. 102-104 °C; ^{1}H NMR (500

MHz, $CDCl_3$) δ 7.11 (dd, J = 10.0 Hz, 6.0 Hz, 1H), 6.25 (d, J = 10.0 Hz, 1H), 5.69 (d, J = 2.5 Hz, 1H), 5.22 (d, J = 2.5 Hz, 1H), 4.71 (d, J = 6.0 Hz, 1H), 4.23 (m, 1H), 3.67 (m, 4H), 3.43 (m, 4H), 3.36 (s, 1H), 2.57 (dt, J = 14.0 Hz, 3.5 Hz, 1H), 2.50 (m, 1H), 2.40 (t, J = 16.0 Hz, 1H), 2.17 (dd, J = 16.0 Hz, 3.5 Hz, 1H), 1.94 (s, 3H), 1.93 (s, 3H), 1.86 (s, 3H), 1.75 -1.59 (m, 5H), 1.41 (m, 2H), 1.36 (s, 3H), 1.12 (d, J =7.5 Hz, 3H), 1.09 (s, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 201.6, 169.7, 162.5, 154.5, 148.5, 141.5, 133.1, 130.8, 122.4, 121.1, 83.7, 81.7, 79.4, 72.8, 61.3, 60.5, 52.2, 48.0, 39.6, 37.5, 35.5, 34.6, 33.1, 32.1, 31.6, 29.9, 29.8, 29.5, 29.4, 27.4, 24.6, 22.9, 21.5, 21.1, 20.6, 17.8, 16.0, 15.7, 14.3, 12.6; HRMS: $[M + Na]^+$ calcd for $C_{35}H_{45}NO_{10}Na$ 662.2936, found 662.2939; purity 99.50% ($MeOH/H_2O$ = 60/40-100/0, R_t = 16.715 min).

(10a). To a solution of the BOC-Glycine (17.5 mg), DMAP (4 mg) and EDCI (200 mg) in anhydrous CH_2Cl_2, add 0.5 mL TEA, and the mixture was stirred at 0 °C for 1h, Then add **1** (26.3 mg), and the mixture was stirred at 25 °C for 24h. **10a** was obtained as a white powder, 20.4 mg, yield 64 %; m.p. 115-117 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.02 (dd, J = 10.0 Hz, 6.0 Hz, 1H), 6.26 (d, J = 10.5 Hz, 1H), 5.68 (d, J = 3 Hz, 1H), 5.21 (d, J = 3 Hz, 1H), 4.94 (t, J = 5.0 Hz, 1H), 4.81 (d, J = 6.0 Hz, 1H), 4.23 (m, 1H), 3.92 (d, J = 5.0 Hz, 2H), 3.35 (brs, 1H), 2.57 (dt, J = 14.0 Hz, 3.0 Hz, 1H), 2.50 (m, 1H), 2.40 (t, J = 16.0 Hz, 1H), 2.17 (dd, J = 16.0 Hz, 3.5 Hz, 1H), 2.04 (s, 1H), 1.94 (s, 3H), 1.93 (s, 3H), 1.86 (s, 3H), 1.83 (m, 1H), 1.75 -1.59 (m, 4H), 1.41 (m, 2H), 1.43 (s, 9H), 1.40 (s, 3H), 1.12 (d, J =7.5 Hz, 3H), 1.08 (s, 3H); ^{13}C NMR (125 MHz, $CDCl_3$), δ 201.6, 169.7, 166.2, 162.5, 155.7, 148.6, 133.7, 122.3, 121.0, 83.7, 81.6, 79.4, 72.6, 61.4, 60.2, 52.1, 48.0, 39.6, 37.4, 35.4, 34.5, 33.0, 24.5, 21.5, 21.2, 20.6, 17.7, 16.0, 14.3, 12.6; HRMS: $[M + NH_4]^+$ calcd for $C_{37}H_{53}N_2O_{11}$ 701.3644, found 701.3644; purity 96.63% ($MeOH/H_2O$ = 75/25, R_t = 6.355 min).

(10b). To a solution of the Boc-L-alanine (18.9 mg), DMAP (4 mg) and EDCI (200 mg) in anhydrous CH_2Cl_2, add 0.5 mL TEA, and the mixture was stirred at 0 °C for 1h, Then add **1** (26.3 mg), and the mixture was stirred at 25 °C for 24h. **10b** was obtained as a white powder, 23.7 mg, yield 68 %; m.p. 96-98 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.02 (dd, J = 10.0 Hz, 6.0 Hz, 1H), 6.26 (d, J = 10.5 Hz, 1H), 5.68 (d, J = 3 Hz, 1H), 5.21 (d, J = 3 Hz, 1H), 5.03 (t, J = 5.0 Hz, 1H), 4.81 (d, J = 6.0 Hz, 1H), 4.23 (m, 1H), 3.38 (m, 1H), 3.37 (brs, 1H), 2.60-2.50 (m, 5H), 2.40 (t, J = 16.0 Hz, 1H), 2.17 (dd, J = 16.0 Hz, 3.5 Hz, 1H), 2.04 (s, 1H), 1.94 (s, 3H), 1.93 (s, 3H), 1.86 (s, 3H), 1.83 (m, 1H), 1.75 -1.59 (m, 4H), 1.43 (s, 9H), 1.41 (m, 2H), 1.40 (s, 3H), 1.12 (d, J =7.5 Hz, 3H), 1.08 (s, 3H); ^{13}C NMR (125 MHz, $CDCl_3$), δ 201.5, 171.7, 169.7, 166.2, 162.5, 155.9, 148.6, 140.2, 133.3, 122.4, 121.0, 83.7, 81.6, 79.6, 79.4, 71.8, 61.8, 60.4, 52.2, 48.0, 39.5, 37.4, 36.3, 35.5, 34.8, 34.5, 33.1, 28.5, 24.6, 21.5, 21.3, 20.6, 17.7, 16.1, 16.0, 12.6; HRMS: $[M + Na]^+$ calcd for $C_{38}H_{51}NO_{11}Na$ 720.3354, found 720.3358; purity 98.88% (MeOH/H_2O = 75/25, R_t = 6.389 min).

(10c). To a solution of the Boc-L-valine (21.7 mg), DMAP (4 mg) and EDCI (200 mg) in anhydrous CH_2Cl_2, add 0.5 mL TEA, and the mixture was stirred at 0 °C for 1h, Then add **1** (26.3 mg), and the mixture was stirred at 25 °C for 24h. **10c** was obtained as a white powder, 24.3 mg, yield 67 % ; m.p. 98-99 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 6.99 (dd, J = 10.0 Hz, 6.0 Hz, 1H), 6.25 (d, J = 10.5 Hz, 1H), 5.68 (d, J = 3 Hz, 1H), 5.22 (d, J = 3 Hz, 1H), 4.84 (dd, J = 23.0 Hz, 6.0 Hz, 1H), 4.29 (m, 1H), 4.23 (m, 1H), 3.35 (brs, 1H), 2.57 (dt, J = 14.0 Hz, 3.5 Hz, 1H), 2.50 (m, 1H), 2.40 (t, J = 16.0 Hz, 1H), 2.17 (dd, J = 16.0 Hz, 3.5 Hz, 1H), 1.93(s, 3H), 1.94 (s, 3H), 1.86 (s, 3H), 1.83 (m, 1H), 1.75 -1.59 (m, 5H), 1.50 (m, 1H), 1.43 (s, 9H), 1.40 (s, 3H), 1.39 (m, 2H),1.12 (d, J =7.5 Hz, 3H), 1.09 (s, 3H), 0.95 (m, 6H); ^{13}C NMR (125 MHz, $CDCl_3$), δ 201.6, 172.8, 169.7, 166.2, 162.5, 148.5, 140.0, 133.4, 122.4, 121.1, 83.7, 81.6, 79.4, 72.0, 61.9, 60.3,

53.6,52.4, 52.2, 47.9, 42.0, 39.7, 37.5, 35.5, 34.6, 33.1, 29.9, 29.5, 28.5, 24.9, 24.6, 23.1, 22.0, 21.6, 21.5, 20.6, 17.7, 16.3, 16.0, 12.6; HRMS: $[M + NH_4]^+$ calcd for $C_{40}H_{59}N_2O_{11}$ 743.4113, found 743.4117; purity 97.18% ($MeOH/H_2O$ = 75/25, R_t = 6.394 min).

(10d). To a solution of the Boc-L-Isoleucine (23.1 mg), DMAP (4 mg) and EDCI (200 mg) in anhydrous CH_2Cl_2, add 0.5 mL TEA, and the mixture was stirred at 0 °C for 1h, Then add **1** (26.3 mg), and the mixture was stirred at 25 °C for 24h. **10d** was obtained as a white powder, 26.2 mg, yield 71 %; m.p. 110-112 °C; ^{1}H NMR (500 MHz, $CHCl_3$) δ 6.99 (dd, J = 10.0 Hz, 6.0 Hz, 1H), 6.25 (d, J = 10.5 Hz, 1H), 5.68 (d, J = 3 Hz, 1H), 5.22 (d, J = 3.0 Hz, 1H), 4.95 (d, J = 10.0 Hz, 1H), 4.84 (d, J = 5.5 Hz, 1H), 4.29 (m, 1H), 4.23 (m, 1H), 3.35 (brs, 1H), 2.57 (dt, J = 14.0 Hz, 3.5 Hz, 1H), 2.50 (m, 1H), 2.40 (t, J = 16.0 Hz, 1H), 2.17 (dd, J = 16.0 Hz, 3.5 Hz, 1H), 1.94 (s, 3H), 1.93 (s, 3H), 1.86 (s, 3H), 1.83 (m, 2H), 1.75 -1.59 (m, 4H), 1.50 (m, 1H), 1.44 (s, 9H), 1.42 (m, 2H), 1.40 (s, 3H), 1.39 (m, 2H), 1.12 (d, J =7.5 Hz, 3H), 1.09 (s, 3H), 0.93 (m, 6H); ^{13}C NMR (125 MHz, $CDCl_3$), δ 201.6, 172.8, 169.7, 166.2, 162.5, 148.5, 139.9, 133.5, 122.4, 121.1, 83.7, 81.6, 79.4, 72.2, 61.8, 60.1, 58.2, 52.2, 47.9, 39.7, 38.2, 37.5, 35.5, 34.6, 33.1, 29.9, 28.5, 25.0, 24.6, 21.5, 20.6, 17.7, 16.2, 16.0, 15.7, 12.6, 11.9; HRMS: $[M + NH_4]^+$ calcd for $C_{41}H_{61}N_2O_{11}$ 757.4270, found 757.4274; purity 96.03% ($MeOH/H_2O$ = 75/25, R_t = 7.444 min).

(10e). To a solution of the rac-Boc Norvaline (21.7 mg), DMAP (4 mg) and EDCI (200 mg) in anhydrous CH_2Cl_2, add 0.5 mL TEA, and the mixture was stirred at 0 °C for 1h, Then add **1** (26.3 mg), and the mixture was stirred at 25 °C for 24h. **10e** was obtained as a white powder, 22.8 mg, yield 73 %; m.p. 109-111 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 6.99 (dd, J = 10.0 Hz, 6.0 Hz, 1H), 6.25 (d, J = 10.5 Hz, 1H), 5.68 (d, J = 3.0 Hz, 1H), 5.22 (d, J = 3.0 Hz, 1H), 4.94 (d, J = 10.0 Hz, 1H), 4.88 (d, J = 5.5 Hz, 1H), 4.26 (m, 1H), 4.23 (m, 1H), 3.37 (brs, 1H), 2.57 (dt, J = 14.0 Hz, 3.5 Hz, 1H), 2.50 (m, 1H), 2.40 (t, J = 16.0 Hz, 1H), 2.22 (d, J = 7.5 Hz, 2H), 2.17 (dd, J = 16.0

Hz, 3.5 Hz, 1H), 2.01 (m, 2H), 1.94 (s, 3H), 1.93 (s, 3H), 1.86 (s, 3H), 1.83 (m, 1H), 1.75 -1.59 (m, 5H), 1.50 (m, 1H), 1.44 (s, 9H), 1.42 (m, 1H), 1.39 (s, 3H), 1.12 (d, J =7.5 Hz, 3H), 1.09 (s, 3H), 0.88 (t, J = 6.5 Hz, 3H); ^{13}C NMR (125 MHz, $CDCl_3$), δ 201.5, 172.5, 169.7, 166.2, 162.5, 148.5, 139.9, 133.5, 130.1, 130.0, 122.4, 121.1, 83.7, 81.6, 79.4, 72.3, 71.2, 61.9, 61.0, 60.3, 52.2, 47.9, 39.7, 37.5, 36.1, 35.5, 34.6, 33.1, 32.1, 31.5, 29.9, 29.9, 29.8, 29.5, 29.4, 28.5, 27.4, 25.7, 24.6, 22.8, 21.5, 20.6, 19.2, 17.7, 17.6, 16.0, 14.3, 12.6; HRMS: $[M + NH_4]^+$ calcd for $C_{40}H_{59}N_2O_{11}$ 743.4113, found 743.4115; purity 95.13% (MeOH/H_2O = 75/25, R_t = 7.441 min).

(10f). To a solution of the *N*-Boc-proline (21.5 mg), DMAP (4 mg) and EDCI (200 mg) in anhydrous CH_2Cl_2, add 0.5 mL TEA, and the mixture was stirred at 0 °C for 1h, Then add **1** (26.3 mg), and the mixture was stirred at 25 °C for 24h. **10f** was obtained as a white powder, 14.8 mg, yield 41 %; m.p. 104-106 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.02 (dd, J = 10.0 Hz, 6.0 Hz, 1H), 6.24 (d, J = 10.5 Hz, 1H), 5.68 (d, J = 3.0 Hz, 1H), 5.22 (d, J = 3.0 Hz, 1H), 4.78 (d, J = 5.5 Hz, 1H), 4.23 (m, 1H), 3.37 (m, 4H), 2.57 (dt, J = 14.0 Hz, 3.5 Hz, 1H), 2.50 (m, 1H), 2.40 (t, J = 16.0 Hz, 1H), 2.22 (d, J = 7.5 Hz, 2H), 2.17 (dd, J = 16.0 Hz, 3.5 Hz, 1H), 2.01 (m, 2H), 1.94 (s, 3H), 1.93 (s, 3H), 1.86 (s, 3H), 1.83 (m, 1H), 1.75 -1.59 (m, 5H), 1.50 (m, 1H), 1.44 (s, 9H), 1.42 (m, 1H), 1.40 (s, 9H), 1.38 (s, 3H), 1.12 (d, J =7.5 Hz, 3H), 1.08 (s, 3H); ^{13}C NMR (125 MHz, $CDCl_3$), δ 201.5, 172.5, 169.6, 166.2, 162.6, 155.7, 148.5, 140.5, 140.2, 133.5, 130.1, 130.0, 122.4, 121.1, 83.7, 81.6, 80.3, 79.4, 72.3, 71.2, 61.9, 61.0, 60.3, 59.2, 59.0, 52.2, 48.1, 46.7, 46.5, 39.6, 37.5, 35.5, 34.6, 33.2, 31.2, 29.9, 28.6, 28.5, 27.4, 25.7, 24.6, 23.7, 21.5, 20.6, 17.8, 16.0, 15.8, 14.3, 12.6; HRMS: $[M + Na]^+$ calcd for $C_{40}H_{53}NO_{11}Na$ 746.3511, found 746.3513; purity 96.92% (MeOH/H_2O = 60-100, R_t = 12.259 min).

(11). To a solution of the 2-thienylacetic acid (14.2 mg), DMAP (4 mg) and EDCI (200 mg) in anhydrous CH_2Cl_2, add 0.5 mL TEA, and the mixture was stirred at 0 °C for 1h, Then add **1** (26.3

mg), and the mixture was stirred at 25 °C for 24h. **11** was obtained as a white powder, 12.0 mg, yield 37 %; m.p. 86-88 ºC; ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.11 (dd, J = 10.0 Hz, 6.0 Hz, 1H), 6.38 (d, J = 15.0 Hz, 1H), 6.26 (d, J = 10.5 Hz, 1H), 6.10 (m, 1H), 5.87 (d, J = 10.0Hz, 1H), 5.69 (d, J = 3.0 Hz, 1H), 5.23 (d, J = 3.0 Hz, 1H), 4.79 (d, J = 6.0 Hz, 1H), 4.23 (m, 1H), 3.37 (brs, 1H), 2.57 (dt, J = 14.0 Hz, 3.0 Hz, 1H), 2.50 (m, 1H), 2.40 (t, J = 16.0 Hz, 1H), 2.17 (dd, J = 16.0 Hz, 3.5 Hz, 1H), 1.94 (s, 3H), 1.93 (s, 3H), 1.86 (s, 3H), 1.83 (m, 1H), 1.75 -1.60 (m, 5H), 1.59 (s, 2H), 1.42 (s, 3H), 1.39 (m, 2H), 1.12 (d, J =7.5 Hz, 3H), 1.09 (s, 3H); ^{13}C NMR (125 MHz, $CDCl_3$), δ 201.4, 169.7, 166.2, 165.3, 162.5, 148.6,140.6, 133.5, 132.1, 127.8, 122.4, 121.1, 83.7, 81.6, 79.4, 72.2, 61.1, 60.4, 52.2, 48.1, 39.7, 37.4, 35.5, 34.6, 33.0, 29.9, 29.5, 27.4, 24.6, 21.5, 21.0, 20.6, 17.7, 16.0, 15.7, 12.6; HRMS: $[M + Na]^+$ calcd for $C_{36}H_{42}O_9Na$, 673.2442, found 673.2445; purity 95.17% (MeOH/H_2O = 60/40-100/0, R_t = 15.069 min).

(12). To a solution of the 3-phenylpropionic acid (15.0 mg), DMAP (4 mg) and EDCI (200 mg) in anhydrous CH_2Cl_2, add 0.5 mL TEA, and the mixture was stirred at 0 °C for 1h, Then add **1** (26.3 mg), and the mixture was stirred at 25 °C for 24h. **12** was obtained as a white powder, 10.3 mg, yield 32 %; m.p. 102-104 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.29 (m, 2H), 7.19 (m, 3H), 7.02 (dd, J = 10.0 Hz, 6.0 Hz, 1H), 6.21 (d, J = 10.5 Hz, 1H), 5.69 (d, J = 3.0 Hz, 1H), 5.22 (d, J = 3.0 Hz, 1H), 4.72 (d, J = 6.0 Hz, 1H), 4.23 (m, 1H), 3.34 (brs, 1H), 2.92 (d, J = 7.5 Hz, 2H), 2.57 (m, 3H), 2.50 (m, 1H), 2.40 (t, J = 16.0 Hz, 1H), 2.17 (dd, J = 16.0 Hz, 3.5 Hz, 1H), 1.94 (s, 3H), 1.93 (s, 3H), 1.86 (s, 3H), 1.83 (m, 1H), 1.75 -1.59 (m, 5H), 1.41 (m, 1H), 1.39 (s, 3H), 1.38 (m, 1H), 1.12 (d, J =7.5 Hz, 3H), 1.08 (s, 3H); ^{13}C NMR (500 MHz, $CHCl_3$), δ 201.4, 172.0, 169.7, 166.2, 162.5, 148.5, 140.6, 140.1, 133.3, 128.7, 128.4, 126.5, 122.4, 121.1, 83.7, 81.7, 79.4, 72.1, 61.1, 60.3, 52.2, 48.0, 39.7, 37.4, 35.8, 34.6, 33.1, 30.9, 29.9, 24.6, 21.5, 21.0, 20.6, 17.7, 16.0, 15.7, 12.6;

HRMS: $[M + Na]^+$ calcd for $C_{39}H_{46}O_9$ 681.3034, found 681.3032; purity 95.67% ($MeOH/H_2O$ = 60/40-100/0, R_t = 17.449 min).

General Procedure for the Synthesis of 13a-13f.

Maleic anhydride was used as the starting material, the corresponding amino unsaturated carboxylic acids were obtained by reaction with different amines, and then react with **1** to get the target compound.

(13a). To a solution of the 4-(dimethylamino)-4-oxobut-2-enoic acid (14.3 mg), DMAP (4 mg) and EDCI (200 mg) in anhydrous CH_2Cl_2, add 0.5 mL TEA, and the mixture was stirred at 0 °C for 1h, Then add **1** (26.3 mg), and the mixture was stirred at 25 °C for 24h. **13a** was obtained as a white powder, 22.1 mg, yield 68 %; m.p. 121-123 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.37 (d, *J* = 13.5 Hz, 1H), 7.07 (dd, *J* = 10.0 Hz, 6.0 Hz, 1H), 6.74 (d, *J* = 13.5 Hz, 1H), 6.27 (d, *J* = 13.5 Hz, 1H), 5.69 (d, *J* = 3.0 Hz, 1H), 5.22 (d, *J* = 3 Hz, 1H), 4.82 (d, J = 6.0 Hz, 1H), 4.23 (m, 1H), 3.38 (brs, 1H), 3.10 (s, 3H), 3.03 (s, 3H), 2.57 (m, 1H), 2.50 (m, 1H), 2.40 (t, J = 16.0 Hz, 1H), 2.17 (dd, J = 16.0 Hz, 3.5 Hz, 1H), 1.94 (s, 3H), 1.93 (s, 3H), 1.86 (s, 3H), 1.83 (m, 1H), 1.75 -1.59 (m, 5H), 1.42 (s, 3H), 1.38 (m, 1H), 1.12 (d, *J* =7.5 Hz, 3H), 1.09 (s, 3H); ^{13}C NMR (125 MHz, $CDCl_3$), δ 201.2, 169.7, 166.3, 164.9, 164.4, 162.6, 148.6, 140.0, 135.1, 133.8, 130.0, 122.4, 121.0, 83.7, 81.6, 79.4, 72.7, 61.2, 60.3, 53.6, 52.2, 48.1, 39.7, 37.6, 35.9, 35.5, 34.5, 33.1, 29.9, 24.5, 22.8, 21.5, 21.0, 20.6, 17.8, 16.0, 15.8, 14.3; HRMS: $[M + Na]^+$ calcd for $C_{36}H_{45}NO_{10}Na$ 674.2936, found 674.2937; purity 97.66% ($MeOH/H_2O$ = 75/25, R_t = 3.169 min).

(13b). To a solution of the 4-(dimethylamino)-4-oxobut-2-enoic acid (14.3 mg), DMAP (4 mg) and EDCI (200 mg) in anhydrous CH_2Cl_2, add 0.5 mL TEA, and the mixture was stirred at 0 °C for 1h, Then add **1** (26.3 mg), and the mixture was stirred at 25 °C for 24h. **13b** was obtained as a

white powder, 19.0 mg, yield 56 %; m.p. 123-125 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.21 (d, *J* = 12.5 Hz, 1H), 7.02 (dd, *J* = 10.0 Hz, 6.0 Hz, 1H), 6.82 (d, *J* = 12.5 Hz, 1H), 6.27 (d, *J* = 10.5 Hz, 1H), 5.69 (d, *J* = 3.0 Hz, 1H), 5.22 (d, *J* = 3.0 Hz, 1H), 4.82 (d, *J* = 6.0 Hz, 1H), 4.23 (m, 1H), 3.55 (q, J = 6.0 Hz, 4H), 3.38 (brs, 1H), 2.57 (dt, *J* = 14.0 Hz, 3.0 Hz, 1H), 2.50 (m, 1H), 2.40 (t, *J* = 16.0 Hz, 1H), 2.17 (dd, *J* = 16.0 Hz, 3.5 Hz, 1H), 2.01 (m, 4H), 1.94 (s, 3H), 1.93 (s, 3H), 1.90 (m, 2H), 1.86 (s, 3H), 1.83 (m, 1H), 1.75 -1.59 (m, 5H), 1.42 (s, 3H), 1.41 (m, 1H), 1.38 (m, 1H), 1.12 (d, *J* =7.5 Hz, 3H), 1.09 (s, 3H); ^{13}C NMR (125 MHz, $CDCl_3$), δ 201.2, 169.7, 166.2, 165.0, 162.5, 162.3, 148.6, 140.0, 135.8, 133.8, 129.8, 122.4, 121.1, 83.7, 81.6, 79.4, 72.6, 61.1, 60.3, 52.2, 48.1, 46.9, 46.4, 39.7, 37.4, 35.5, 34.5, 33.1, 26.2, 24.5, 24.4, 21.5, 21.0, 20.6, 17.7, 16.0, 15.8, 12.6; HRMS: $[M + Na]^+$ calcd for $C_{38}H_{47}NO_{10}Na$ 702.3249, found 702.3247; purity 97.14% (MeOH/H_2O = 75/25, R_t = 3.295 min).

(13c). To a solution of the 4-(diisopropylamino)-4-oxobut-2-enoic acid (19.9 mg), DMAP (4 mg) and EDCI (200 mg) in anhydrous CH_2Cl_2, add 0.5 mL TEA, and the mixture was stirred at 0 °C for 1h, Then add **1** (26.3 mg), and the mixture was stirred at 25 °C for 24h. **13c** was obtained as a white powder, 22.6 mg, yield 64 %; m.p. 124-126 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.34 (d, *J* = 13.5 Hz, 1H), 7.08 (dd, *J* = 10.0 Hz, 6.0 Hz, 1H), 6.60 (d, *J* = 13.5 Hz, 1H), 6.27 (d, *J* = 10.5 Hz, 1H), 5.69 (d, *J* = 3.0 Hz, 1H), 5.22 (d, *J* = 3.0 Hz, 1H), 4.81 (d, *J* = 6.0 Hz, 1H), 4.23 (m, 1H), 3.93 (m, 2H), 3.37 (brs, 1H), 2.57 (dt, *J* = 14.0 Hz, 3.0 Hz, 1H), 2.50 (m, 1H), 2.40 (t, *J* = 16.0 Hz, 1H), 2.17 (dd, *J* = 16.0 Hz, 3.5 Hz, 1H), 1.94 (s, 3H), 1.93 (s, 3H), 1.86 (s, 3H), 1.83 (m, 1H), 1.75 - 1.59 (m, 5H), 1.42 (s, 3H), 1.41 (m, 1H), 1.38 (s, 3H), 1.37 (s, 3H), 1.25 (s, 3H), 1.24 (s, 3H), 1.12 (d, *J* =7.5 Hz, 3H), 1.08 (s, 3H); ^{13}C NMR (125 MHz, $CDCl_3$), δ 201.2, 169.7, 166.2, 165.1, 164.0, 162.5, 148.6, 140.1, 138.7, 133.7, 128.1, 122.4, 121.0, 83.7, 81.6, 79.4, 72.6, 61.1, 60.3, 53.6, 52.2, 48.0, 39.7, 37.4, 35.5, 34.6, 33.1, 24.6, 21.5, 17.7, 17.8, 16.0, 15.9, 15.8, 12.6; HRMS: $[M + Na]^+$

calcd for $C_{40}H_{53}NO_{10}Na$ 730.3562, found 730.3560; purity 95.62% (MeOH/H_2O = 75/25, R_t = 2.990 min).

(13d). To a solution of the 4-oxo-4-(pyrrolidin-1-yl) but-2-enoic acid (16.9 mg), DMAP (4 mg) and EDCI (200 mg) in anhydrous CH_2Cl_2, add 0.5 mL TEA, and the mixture was stirred at 0 °C for 1h, Then add **1** (26.3 mg), and the mixture was stirred at 25 °C for 24h. **13d** was obtained as a white powder, 15.9 mg, yield 47 %; m.p. 126-128 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.38 (d, *J* = 10.5 Hz, 1H), 7.08 (dd, *J* = 10.0 Hz, 6.0 Hz, 1H), 6.68 (d, *J* = 10.5 Hz, 1H), 6.28 (d, *J* = 7.5 Hz, 1H), 5.69 (d, *J* = 3.0 Hz, 1H), 5.23 (d, J = 3.0 Hz, 1H), 4.82 (d, J = 6.0 Hz, 1H), 4.23 (m, 1H), 3.62(t, *J* = 5.0 Hz, 1H), 3.47(t, *J* = 5.0 Hz, 1H), 3.38 (brs, 1H), 2.57 (dt, *J* = 14.0 Hz, 3.0 Hz, 1H), 2.50 (m, 1H), 2.40 (t, *J* = 16.0 Hz, 1H), 2.17 (dd, *J* = 16.0 Hz, 3.5 Hz, 1H), 2.04 (s, 2H), 1.94 (s, 3H), 1.93 (s, 3H), 1.86 (s, 3H), 1.83 (m, 1H), 1.75 -1.59 (m, 5H), 1.42 (s, 3H), 1.38-1.27(m, 5H), 1.12 (d, *J* =7.5 Hz, 3H), 1.09 (s, 3H); ^{13}C NMR (125 MHz, $CDCl_3$), δ 201.2, 169.7, 166.2, 165.0, 163.1, 162.5, 148.5, 140.0, 136.0, 133.8, 129.5, 122.4, 121.1, 83.7 81.6, 79.4, 72.6, 61.2, 60.3, 53.6, 52.2, 48.0, 47.4, 43.4, 39.7, 37.4, 35.5, 34.5, 33.1, 26.8, 25.6, 24.6, 22.9, 21.5, 21.02, 20.6, 17.7, 16.0, 15.8, 14.3, 12.6; HRMS: $[M + Na]^+$ calcd for $C_{38}H_{47}NO_{10}Na$ 700.3092, found 700.3097; purity 95.21% (MeOH/H_2O = 75/25, R_t = 5.243 min).

(13e). To a solution of the 4-oxo-4-(piperidin-1-yl)but-2-enoic acid (18.3 mg), DMAP (4 mg) and EDCI (200 mg) in anhydrous CH_2Cl_2, add 0.5 mL TEA, and the mixture was stirred at 0 °C for 1h, Then add **1** (26.3 mg), and the mixture was stirred at 25 °C for 24h. **13e** was obtained as a white powder, 21.1 mg, yield 61 %; m.p. 114-116 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.06 (dd, *J* = 10.0 Hz, 6.0 Hz, 1H), 6.60 (d, *J* = 10.5 Hz, 1H), 6.24 (d, *J* = 7.5 Hz, 1H), 5.98 (d, *J* = 10.5 Hz, 1H), 5.68 (d, *J* = 3 Hz, 1H), 5.21 (d, *J* = 3 Hz, 1H), 4.80 (d, *J* = 6.0 Hz, 1H), 4.23 (m, 1H), 3.45 (m, 1H), 3.34 (brs, 1H), 3.25 (m, 1H), 2.57 (dt, *J* = 14.0 Hz, 3.0 Hz, 1H), 2.50 (m, 1H), 2.40 (t, *J*

= 16.0 Hz, 1H), 2.17 (dd, *J* = 16.0 Hz, 3.5 Hz, 1H), 2.04 (s, 2H), 1.94 (s, 3H), 1.93 (s, 3H), 1.86 (s, 3H), 1.83 (m, 1H), 1.75 -1.59 (m, 5H), 1.39 (s, 3H), 1.38 (m, 1H), 1.25-1.18 (m, 6H), 1.12 (d, J =7.5 Hz, 3H), 1.08 (s, 3H); ^{13}C NMR (125 MHz, $CDCl_3$), δ 201.4, 171.3, 169.7, 166.2, 165.9, 163.7, 162.5, 148.6, 140.3, 139.9, 133.6, 122.4, 121.9, 121.0, 83.7, 81.6, 79.4, 72.2, 61.2, 60.6, 60.2, 52.2, 48.0, 42.6, 39.7, 39.0, 37.4, 35.5, 34.5, 33.1, 24.6, 21.5, 21.2, 21.1, 20.6, 17.7, 16.0, 15.9, 14.4, 14.2, 12.7, 12.6; HRMS: $[M + Na]^+$ calcd for $C_{39}H_{49}NO_{10}Na$ 714.3249, found 714.3251; purity 95.49% ($MeOH/H_2O$ = 75/25, R_t = 4.524 min).

(13f). To a solution of the 4-morpholino-4-oxobut-2-enoic acid (18.5 mg), DMAP (4 mg) and EDCI (200 mg) in anhydrous CH_2Cl_2, add 0.5 mL TEA, and the mixture was stirred at 0 °C for 1h, Then add **1** (26.3 mg), and the mixture was stirred at 25 °C for 24h. **13f** was obtained as a white powder, 24.3 mg, yield 70 %; m.p. 106-108 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.32 (d, *J* = 10.5 Hz, 1H), 7.08 (dd, *J* = 10.0 Hz, 6.0 Hz, 1H), 6.76 (d, *J* = 10.5 Hz, 1H), 6.28 (d, *J* = 7.5 Hz, 1H), 5.69 (d, *J* = 3 Hz, 1H), 5.23 (d, *J* = 3 Hz, 1H), 4.82 (d, *J* = 6.0 Hz, 1H), 4.23 (m, 1H), 3.70 (m, 6H), 3.35(m, 2H), 3.38 (brs, 1H), 2.57 (dt, *J* = 14.0 Hz, 3.0 Hz, 1H), 2.50 (m, 1H), 2.40 (t, J = 16.0 Hz, 1H), 2.17 (dd, J = 16.0 Hz, 3.5 Hz, 1H), 2.04 (s, 1H), 1.94 (s, 3H), 1.93 (s, 3H), 1.86 (s, 3H), 1.83 (m, 1H), 1.75 -1.59 (m, 5H), 1.41 (s, 3H), 1.38 (m, 1H), 1.12 (d, *J* =7.5 Hz, 3H), 1.09 (s, 3H); ^{13}C NMR (125 MHz, $CDCl_3$), δ 201.2, 169.6, 166.2, 164.7, 163.3, 162.6, 148.5, 139.8, 134.4, 133.9, 130.7, 122.4, 121.0, 83.7, 81.6, 79.5, 72.8, 66.9, 61.2, 60.3, 52.2, 48.1, 46.6, 42.6, 39.7, 37.4, 35.5, 34.5, 33.2, 24.6, 21.5, 21.1, 20.6, 17.8, 16.0, 15.9, 12.6; HRMS: $[M + Na]^+$ calcd for $C_{38}H_{47}NO_{10}Na$ 716.3041, found 716.3043; purity 95.84% ($MeOH/H_2O$ = 75/25, R_t = 4.160 min).

(**13a'**). To a solution of the 4-(dimethylamino)-4-oxobutanoic acid (14.5 mg), DMAP (4 mg) and EDCI (200 mg) in anhydrous CH_2Cl_2, add 0.5 mL TEA, and the mixture was stirred at 0 °C for

1h, then, **1** (26.3 mg) was added, and the mixture was stirred at 25 °C for 24h. **13a'** was obtained as a white powder, 24.5 mg, yield 75 %; m.p. 101-102 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ7.05 (dd, *J* = 10.0 Hz, 6.0 Hz, 1H), 6.24 (d, *J* = 13.5 Hz, 1H), 5.68 (d, *J* = 3.0 Hz, 1H), 5.22 (d, *J* = 3 Hz, 1H), 4.78 (d, J = 6.0 Hz, 1H), 4.23 (m, 1H), 3.38 (brs, 1H), 3.05 (s, 3H), 3.00 (s, 3H), 2.68 (m, 4H), 2.57 (m, 1H), 2.50 (m, 1H), 2.40 (t, J = 16.0 Hz, 1H), 2.17 (dd, J = 16.0 Hz, 3.5 Hz, 1H), 1.94 (s, 3H), 1.93 (s, 3H), 1.86 (s, 3H), 1.83 (m, 1H), 1.75 -1.59 (m, 5H), 1.42 (s, 3H), 1.38 (m, 1H), 1.12 (d, *J* =7.5 Hz, 3H), 1.09 (s, 3H); ^{13}C NMR (125 MHz, $CDCl_3$), δ 201.3, 172.1, 170.7, 169.6, 166.1, 162.4, 148.4, 140.5, 133.1, 122.3, 120.1, 83.6, 81.5, 79.2, 71.8, 61.2, 60.3, 52.1, 47.9, 39.6, 37.3, 37.0, 35.5, 35.3, 34.5, 33.0, 31.45, 29.4, 28.4, 28.1, 24.5, 21.3, 21.0, 20.4, 17.6, 15.9, 15.7, 12.5; HRMS: $[M + Na]^+$ calcd for $C_{36}H_{47}NO_{10}Na$ 676.3092, found 676.3096; purity 95.26% ($MeOH/H_2O$ = 75/25, R_t = 3.496 min).

Cytotoxicity assays

HT-29, HCT-116, HepG2 MCF-7, L02 and HK-2 cells were obtained from the Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences and were cultured in DMEM or RPMI supplemented with 10% FBS, 2 mM glutamine, and 100 units/mL penicillin/streptomycin and maintained in a humidified atmosphere of 5% CO_2 at 37ºC.

MTT assay

HT-29, HCT-116, HepG2, MCF-7, L02 and HK-2 cells were seeded in 96-well plates and then treated with vehicle alone or with the tested compounds for 24 h. Then, 20 μL of MTT (5 mg/mL, in PBS) was added to each well, and the samples were further incubated for 4 h. The MTT formazan formed by viable cells was dissolved in DMSO (150 μL), and absorbance was measured using a microplate reader (570 nm).

Measurement of *in vitro* TrxR activity by DTNB Assay[39]

TrxR activity was determined at room temperature using a microplate reader. NADPH-reduced TrxR (0.16 μM) was incubated with different concentrations of compounds for different time at room temperature (the final volume of the mixture was 50 μL) in a 96-well plate. A master mixture in TE buffer (50 μL) containing DTNB and NADPH was added (final concentration of 2 mM and 200 μM, respectively), and the linear increase in absorbance at 412 nm during the initial 3 min was recorded. The same amounts of DMSO (0.1%, v/v) were added to the control experiments, and the activity was expressed as the percentage of the control.

The K_{inact} and K_i through time-course experiments

TrxR activity was determined at room temperature using a microplate reader. Different concentrations of **WA** and **13a** were incubated in TrxR for 0-30 min, residual enzyme activity was measured by DTNB Assay.

Measurement of in vitro Trx activity by insulin reduction assay

Trx activity was determined at room temperature using a microplate reader. E. coli Trx (5.0 μM) was incubated with different concentrations of WA or 13a for 1.5 h at room temperature (the final volume of the mixture was 50 μL) in a 96-well plate. A master mixture in TE buffer (50 μL) containing insulin and DTT (final concentrations of 0.17 mM and 2.0 mM, respectively) was added. The decrease in absorbance (A340) was recorded immediately after the initial 1-min analysis of a blank reference.

Determination of TrxR, Trx, GR and GPx activity in HT-29 cells

After HT-29 cells were treated with different concentrations of **13a** for 24 h. The cells were harvested and washed twice with PBS. Total cellular proteins were extracted by RIPA buffer (50 mM Tris-HCl, pH 7.5, 2 mM EDTA, 0.5% deoxycholate, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 1 mM Na_3VO_4, and 1 mM PMSF) for 30 min on ice. Total protein content was quantified using the BCA procedure. TrxR activity in cell lysates was measured by the end point insulin

reduction assay. Briefly, the cell extract containing 50 μg of total protein was incubated in a final reaction volume of 50 μL containing 100 mM Tris-HCl (pH 7.6), 0.3 mM insulin, 660 μM NADPH, 3 mM EDTA, and 15 μM E. coli Trx for 30 min at 37ºC. The reaction was terminated by adding 200 μL of 1 mM DTNB in 6 M guanidine hydrochloride, pH 8.0. A blank sample, containing everything except Trx, was treated in the same manner. The absorbance at 412 nm was measured, and the blank value was subtracted from the corresponding absorbance value of the sample. The same amounts of DMSO were added to the control experiments, and the activity was expressed as the percentage of the control. Trx activity in cell lysates was measured by the end point insulin reduction assay. Cell extract containing 50 μg of total protein was incubated in TE buffer (50 μL) containing insulin and DTT (final concentrations of 0.17 mM and 2.0 mM, respectively). The decrease in absorbance (A340) was recorded immediately after the initial 1-min analysis of a blank reference. GR assay. Cell extract containing 50 μg of total protein was incubated in TE buffer (50 mM Tris-HCl with 1 mM EDTA, pH 7.5), and oxidized glutathione (GSSG) and NADPH (50 μL, final concentration of 1 mM and 400 μM, respectively) was added. GR activity was determined by measuring the decrease in absorbance at 340 nm during the initial 3 min. The same amounts of DMSO were added to the control experiments, and the activity was expressed as the percentage of the control. GPx activity was measured indirectly by a coupled reaction with GR. GSSG, produced by the reduction of hydroperoxides by GPx, is recycled to its reduced state by GR and NADPH. The oxidation of NADPH to $NADP^+$ is accompanied by a decrease in absorbance at 340 nm. The rate of decrease in A340 is directly proportional to GPx activity. To the wells of a 96-well microliter plate, 130 μL of TE buffer, 10 μL of a freshly prepared NADPH solution (4.0 mM in TE buffer), and 50 μg of total protein were added. Then, 10 μL of a baker's yeast GR solution and 10 μL of freshly prepared GSH solution (5.0 mM in TE buffer) were

added. After the addition of 20 μL of an H_2O_2 solution (5.0 mM in water), the final volume in each well was 200 μL. The background of the GPx-independent NADPH oxidation rate (r1) was determined by replacing the GPx solution with TE buffer. The rate of decrease in the absorption of NADPH at 340 nm (r2) was measured for 4 min at intervals of 10 s at room temperature. The relative GPx activity was calculated by subtracting the r1 from r2 and was expressed as the percentage of the control.

Analysis of the mass spectrum of 13a-treated and untreated TrxR

Reduced-rat liver TrxR (1.0 μM) was incubated with **13a** (10.0 μM) at room temperature. After 1.5 h, the **13a**-modified protein was purified by SDS−PAGE and digested with trypsin in the gel. The resulting peptides were analyzed using LC-MS/MS.

Mercapto binding experiments

NAC (10mg) were incubated with **13a** (5mg) at room temperature, and the online NMR analysis was developed. DTT (10mg) and NAC (10mg) were incubated with **13a** (5mg) and **WA**(5mg) at room temperature. After 1.5 h, the reaction liquid was analyzed using LC-MS/MS.

Determination of intracellular ROS

To assay the intracellular accumulation of ROS, HT-29 or HCT-116 cells (3.0×10^5 cells/well) were seeded into 6-well plates. After 24 h, the cells were treated with different concentrations of 13a or GSH (5 mM) for an additional 24 h. The cells were collected, washed with PBS, and stained with DCFH-DA. ROS were determined by flow cytometry.

Monoclonal formation experiment

HT-29 cells were incubated in six-well plates (5×10^2/well) for 12 h and then treated with DMSO (1%) or various concentrations (0.0125, 0.03, 0.06 μM) of 13a for 24 h. The added medium was removed and replaced with normal fresh medium, and the solution was changed every 2-3 days.

The formation of clones was observed under an optical microscope. After 14 days, the culture was terminated when visible clones appeared in the 6-well plates. The culture solution was discarded, washed with PBS 2 times, and added to each hole 4% paraformaldehyde was fixed for 30 min. The fixing solution was discarded, 1 mL crystal violet staining solution was added to each hole for 20 min, and then PBS was used slowly wash away the stain and let dry in the air before taking photos.

EdU infiltration experiment

According to the manual for the EdU labeling/detection kit (Ribobio, Guangzhou, China), HT-29 cells were incubated in a final volume of 100 μL of complete medium at 5.0×10^3 cells per well in 96-well plates. Following incubation for 24 h, the cells were replaced with fresh medium containing 13a at different concentrations (0.125-0.500 μM) or DMSO as a vehicle (< 0.1%). After 20 h, 100 μL of fresh medium containing 50 mM EdU labeling agent was added to the cell culture to incubate for an additional 8 h at 37 ºC under 5% CO^2. The cells were then fixed with 4% paraformaldehyde (pH 7.4) for 30 min and incubated with glycine for 5 min. After washing with PBS, the cells were stained with anti-EdU working solution at room temperature for 30 min. After washing with 0.5% Triton X-100 in PBS, the cells were incubated with 5 μg mL^{-1} Hoechst 33258 at room temperature for 30 min and then observed under a High Content Imaging System (Image Xpress Micro Confocal, Molecular Devices).

Induced G2/M arrest in HT-29 cells

HT-29 cells were incubated in six-well plates (3.0×10^5/ well) and treated with DMSO (1%) or various concentrations (0.125, 0.25, 0.5 μM) of **13a** for 24 h. The cells were collected, washed with PBS, fixed in ice-chilled 70% EtOH for 12 h and incubated again for 30 min at 37 ºC in a

staining and stained with RNaseA and PI (Beyotime, Nantong, People's Republic of China). Mitochondrial membrane potential was determined by flow cytometry.

Apoptosis analysis

HT-29 cells were incubated in six-well plates (3.0×10^5/well) and treated with DMSO (1%) or **13a** for 24 h. The cells were collected, washed with PBS, and stained with FITC-Annexin-V and PI. Apoptosis was determined by flow cytometry.

Effect of 13a on the mitochondrial membrane potential of cells

The mitochondrial membrane potential (M.M.P) was determined by a JC-1 assay kit (Nanjing KeyGen Biotech Co., Ltd., Nanjing, China). After collection, MG-63 cells were washed with PBS twice, supplemented with 500 mL of JC-1 dye staining solution, and then incubated in the dark at 37 ºC for 25 min. After incubation, the cells were centrifuged at 2000 rpm for 5 min and washed twice with 1× incubation buffer. The fluorescence was then detected using flow cytometry (488 nm excitation and 525 nm emission filters) after the cells were resuspended in 500 μL of 1× incubation buffer.

Western blot analysis

HT-29 cells were incubated with various concentrations of **13a** or 0.1% DMSO for 24 h. Harvested after trypsinization, cells were treated with 1×RIPA lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.25% deoxycholic acid, 1% NP-40, 1 mM EDTA and protease inhibitors) (Amresco, Solon, USA) to extract the total proteins. An aliquot of proteins from the total cell lysates (30 to 40 μg per lane) was separated by sodium dodecyl sulfate (8%, 12% or 15%) polyacrylamide gel electrophoresis (SDS-PAGE, BioRad Laboratories, Hercules, CA), wet-transferred to PVDF membrane (BioRad Laboratories, Hercules, CA), blotted with primary antibodies specific for TrxR, Trx, ASK1, p-ASK1, P53, Bcl-2, Bax, PARP, cleaved-PARP,

cleaved-caspase-3, caspase-3, and GAPDH, and probed with secondary isotype-specific antibodies tagged with horseradish peroxidase (Cell Signaling Technology). Bound immunocomplexes were detected using a ChemiDOC™ XRS + system (BioRad Laboratories, Hercules, CA).

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at xxxx.

Supplementary figures, NMR spectra, analytical HPLC traces and small molecule X-ray crystal structure for **3.**

Molecular formula strings with pharmacological data (CSV)

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Notes

The authors have no conflicts of interest to declare.

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ABBREVIATIONS

DCFH, dichlorodihydrofluorescin; DCFH-DA, 2',7'-dichlorofluorescin diacetate; DTT, DL-dithiothreitol; FITC, fluorescein 5-isothiocyanate; GPx, glutathione peroxidase; GR, glutathione reductase; GSH, reduced glutathione; GSSG, oxidized glutathione; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2Htetrazoliumbromide; NAC, N-acetylcysteine; PI, propidium iodide; ROS, reactive oxygen species; Sec, selenocysteine; TE buffer, 50 mM Tris-HCl with 1 mM EDTA, pH 7.5; Trx, thioredoxin; TrxR, thioredoxin reductase.

REFERENCES

(1) Lee, A. C.; Fenster, B. E.; Ito, H.; Takeda, K.; Bae, N. S.; Hirai, T.; Yu, Z. X.; Ferrans, V. J.; Howard, B. H.; Finkel, T. Ras proteins induce senescence by altering the intracellular levels of reactive oxygen species. *J. Biol. Chem.* **1999,** *274*, 7936-7940.

(2) Cross, C. E.; Halliwell, B.; Borish, E. T.; Pryor, W. A.; Ames, B. N.; Saul, R. L.; McCord, J. M.; Harman, D. Oxygen radicals and human disease. *Ann. Intern. Med.* **1987,** *107*, 526-545.

(3) Wu, W. S. The signaling mechanism of ROS in tumor progression. *Cancer Metastasis Rev.* **2006,** *25*, 695-705.

(4) Gorrini, C.; Harris, I. S.; Mak, T. W. Modulation of oxidative stress as an anticancer strategy. *Nat. Rev. Drug Discovery* **2013,** *12*, 931-947.

(5) Zhang, J.; Li, X.; Han, X.; Liu, R.; Fang, J. Targeting the thioredoxin system for cancer therapy. *Trends Pharmacol. Sci.* **2017,** *38*, 794-808.

(6) Lu, J.; Holmgren, A. The thioredoxin antioxidant system. *Free Radical Biol. Med.* **2014,** *66*, 75-87.

(7) Mahmood, D. F.; Abderrazak, A.; El Hadri, K.; Simmet, T.; Rouis, M. The thioredoxin system as a therapeutic target in human health and disease. *Antioxid. Redox Signaling* **2013,** *19*, 1266-1303.

(8) Bindoli, A.; Rigobello, M. P. Principles in redox signaling: From chemistry to functional significance. *Antioxid. Redox Signaling* **2013,** *18*, 1557-1593.

(9) Zhong, L.; Arner, E. S.; Holmgren, A. Structure and mechanism of mammalian thioredoxin reductase: The active site is a redox-active selenolthiol/selenenylsulfide formed from the conserved cysteine-selenocysteine sequence. *Proc. Natl. Acad. Sci. U. S. A.* **2000,** *97*, 5854-5859.

(10) Sandalova, T.; Zhong, L.; Lindqvist, Y.; Holmgren, A.; Schneider, G. Three-dimensional structure of a mammalian thioredoxin reductase: Implications for mechanism and evolution of a selenocysteine-dependent enzyme. *Proc. Natl. Acad. Sci. U. S. A.* **2001,** *98*, 9533-9538.

(11) Fang, J.; Holmgren, A. Inhibition of thioredoxin and thioredoxin reductase by 4-hydroxy-2-nonenal in vitro and in vivo. *J. Am. Chem. Soc.* **2006,** *128*, 1879-1885.

(12) Kahlos, K.; Soini, Y.; Saily, M.; Koistinen, P.; Kakko, S.; Paakko, P.; Holmgren, A.; Kinnula, V. L. Up-regulation of thioredoxin and thioredoxin reductase in human malignant pleural mesothelioma. *Int. J. Cancer* **2001,** *95*, 198-204.

(13) Berggren, M.; Gallegos, A.; Gasdaska, J. R.; Gasdaska, P. Y.; Warneke, J.; Powis, G. Thioredoxin and thioredoxin reductase gene expression in human tumors and cell lines, and the effects of serum stimulation and hypoxia. *Anticancer Res.* **1996,** *16*, 3459-3466.

(14) Fang, J.; Lu, J.; Holmgren, A. Thioredoxin reductase is irreversibly modified by curcumin: A novel molecular mechanism for its anticancer activity. *J. Biol. Chem.* **2005,** *280*, 25284-25290.

(15) Kingston, D. G. Modern natural products drug discovery and its relevance to biodiversity conservation. *J. Nat. Prod.* **2011,** *74*, 496-511.

(16) Koehn, F. E.; Carter, G. T. The evolving role of natural products in drug discovery. *Nat. Rev. Drug Discovery* **2005,** *4*, 206-220.

(17) Juang, J. K.; Huang, H. W.; Chen, C. M.; Liu, H. J. A new compound, Withangulatin A, promotes type II DNA topoisomerase-mediated DNA damage. *Biochem. Biophys. Res. Commun.* **1989,** *159*, 1128-1134.

(18) Lin, Y. S.; Chiang, H. C.; Kan, W. S.; Hone, E.; Shih, S. J.; Won, M. H. Immunomodulatory activity of various fractions derived from *Physalis angulata* L extract. *Am. J. Chin. Med.* **1992,** *20*, 233-243.

(19) Chen, C. M.; Chen, Z. T.; Hsieh, C. H.; Li, W. S.; Wen, S. Y. Withangulatin A, a new withanolide from *Physalis angulate*. *Heterocycles*, **1990**, *31*, 1371–1375.

(20) Lee, W. C.; Lee, Y. C.; Peng, M. D.; Chen, C. M.; Lai, Y. K. Induction of vimentin modification and vimentin-HSP72 association by withangulatin A in 9L rat brain tumor cells. *J. Cell. Biochem*., **1993**, 52, 253–265.

(21) Ding, W.; Hu, Z.; Zhang, Z.; Ma, Q.; Tang, H.; Ma, Z. Physapubescin B exhibits potent activity against human prostate cancer in vitro and in vivo. *J. Agric. Food Chem.* **2015,** *63*, 9504-9512.

(22) Wijeratne, E. M. K.; Xu, Y.M.; Scherz-Shouval, R.; Marron, M. T.; Rocha, D. D.; Liu, M. X.; Costa-Lotufo, L. V.; Santagata, S.; Lindquist, S.; Whitesell, L.; Gunatilaka, A. A. L. Structure–activity relationships for withanolides as inducers of the cellular heat-shock response. *J. Med. Chem.* **2014,** *57*, 2851-2863.

(23) Ishiguro, M.; Kajikawa, A.; Haruyama, T.; Morisaki, M.; Ikekawa, N. Synthetic studies of withanolide I synthesis of ab Ring moiety of withaferin A. *Tetrahedron Lett.* **1974,** *15*, 1421-1424.

(24) Zhang, H.; Samadi, A. K.; Cohen, M. S.; Timmermann, B. N. Anti-proliferative withanolides from the solanaceae: A structure-activity study. *Pure Appl. Chem.* **2012,** *84*, 1353-1367.

(25) Xu, Y. M.; Liu, M. X.; Grunow, N.; Wijeratne, E. M. K.; Paine-Murrieta, G.; Felder, S.; Kris, R. M.; Gunatilaka, A. A. L. Discovery of potent 17β-hydroxywithanolides for castration-resistant prostate cancer by high-throughput screening of a natural products library for androgen-induced gene expression inhibitors. *J. Med. Chem.* **2015,** *58*, 6984-6993.

(26) Xu, Y. M.; Brooks, A. D.; Wijeratne, E. M.; Henrich, C. J.; Tewary, P.; Sayers, T. J.; Gunatilaka, A. A. 17beta-hydroxywithanolides as sensitizers of renal carcinoma cells to tumor necrosis factor-alpha related apoptosis inducing ligand (trail) mediated apoptosis: Structure-activity relationships. *J. Med. Chem.* **2017,** *60*, 3039-3051.

(27) Lenci, E.; Trabocchi, A. Peptidomimetic toolbox for drug discovery. *Chem. Soc. Rev.* **2020,** *49*, 3262-3277.

(28) Duan, D.; Zhang, B.; Yao, J.; Liu, Y.; Sun, J.; Ge, C.; Peng, S.; Fang, J. Gambogic acid induces apoptosis in hepatocellular carcinoma SMMC-7721 cells by targeting cytosolic thioredoxin reductase. *Free Radical Biol. Med.* **2014,** *69*, 15-25.

(29) Duan, D.; Zhang, B.; Yao, J.; Liu, Y.; Fang, J. Shikonin. Targets cytosolic thioredoxin reductase to induce ROS-mediated apoptosis in human promyelocytic leukemia HL-60 cells. *Free Radical Biol. Med.* **2014,** *70*, 182-193.

(30) Cai, W.; Zhang, B.; Duan, D.; Wu, J.; Fang, J. Curcumin targeting the thioredoxin system elevates oxidative stress in Hela cells. *Toxicol. Appl. Pharmacol.* **2012,** *262*, 341-348.

(31) Wang, X.; Shen, Y.; Wang, S.; Li, S.; Zhang, W.; Liu, X.; Lai, L.; Pei, J.; Li, H. PharmMapper 2017 Update: A web server for potential drug target identification with a comprehensive target pharmacophore database. *Nucleic Acids Res.* **2017,** *45*, W356-W360.

(32) Jan, Y. H.; Heck, D. E.; Dragomir, A. C.; Gardner, C. R.; Laskin, D. L.; Laskin, J. D. Acetaminophen reactive intermediates target hepatic thioredoxin reductase. *Chem. Res. Toxicol.* **2014,** *27*, 882-894.

(33) Nordberg, J.; Zhong, L.; Holmgren, A.; Arner, E. S. Mammalian thioredoxin reductase is irreversibly inhibited by dinitrohalobenzenes by alkylation of both the redox active selenocysteine and its neighboring cysteine residue. *J. Biol. Chem.* **1998,** *273*, 10835-10842.

(34) Liu, Y.; Min, W. Thioredoxin promotes ASK1 ubiquitination and degradation to inhibit ASK1-mediated apoptosis in a redox activity-independent manner. *Circ. Res.* **2002,** *90*, 1259-1266.

(35) Tonissen, K. F.; Di Trapani, G. Thioredoxin system inhibitors as mediators of apoptosis for cancer therapy. *Mol. Nutr. Food Res.* **2009,** *53*, 87-103.

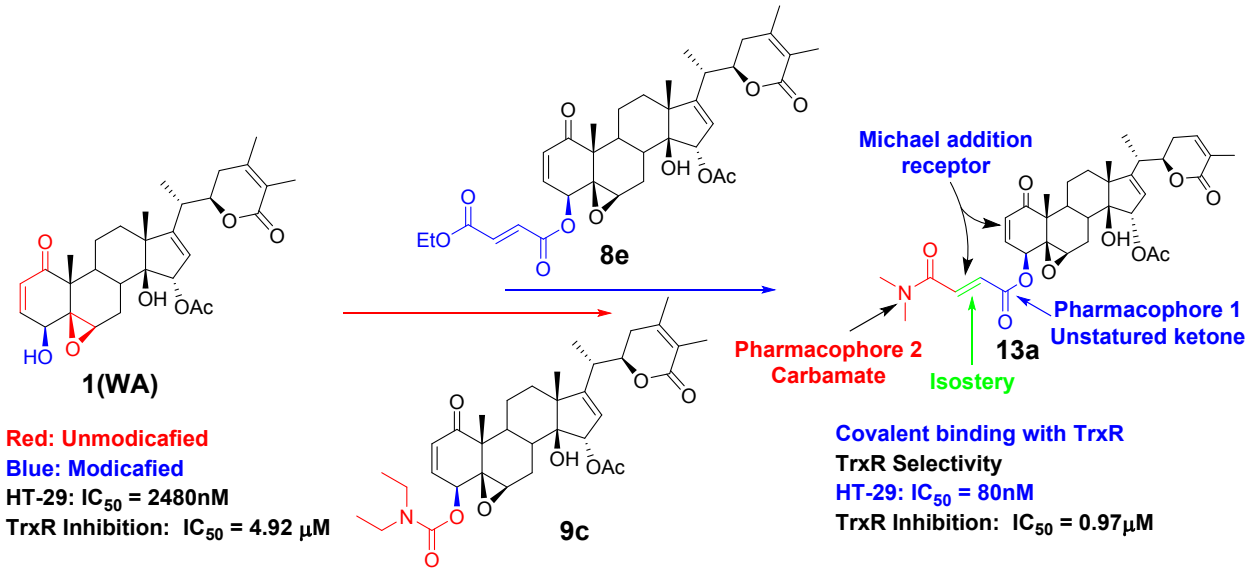
(36) Laurent, T. C.; Moore, E. C.; Reichard, P. Enzymatic synthesis of deoxyribonucleotides. IV. isolation and characterization of thioredoxin, the hydrogen donor from *Escherichia coli* B. *J. Biol. Chem.* **1964,** *239*, 3436-3444.

(37) Chae, H. Z.; Chung, S. J.; Rhee, S. G. Thioredoxin-dependent peroxide reductase from yeast. *J. Biol. Chem.* **1994,** *269*, 27670-27678.

(38) Gonzalez Porque, P.; Baldesten, A.; Reichard, P. The involvement of the thioredoxin system in the reduction of methionine sulfoxide and sulfate. *J. Biol. Chem.* **1970,** *245*, 2371-2374.

(39) Zhang, B.; Duan, D.; Ge, C.; Yao, J.; Liu, Y.; Li, X.; Fang, J. Synthesis of xanthohumol analogues and discovery of potent thioredoxin reductase inhibitor as potential anticancer agent. *J. Med. Chem.* **2015,** *58*, 1795-1805.

TOC GRAPHIC


1(WA)
Red: Unmodicafied
Blue: Modicafied
HT-29: IC50 = 2480nM
TrxR Inhibition: IC50 = 4.92 μM
8e
9c
Michael addition receptor
Pharmacophore 2
Carbamate
Isostery
13a
Pharmacophore 1
Unstatured ketone
Covalent binding with TrxR
TrxR Selectivity
HT-29: IC50 = 80nM
TrxR Inhibition: IC50 = 0.97μM