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Original article

The synthesis and biological evaluation of novel Danshensu–cysteine analog conjugates as cardiovascular-protective agents

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ABSTRACT

A series of novel amide and thioester conjugates between Danshensu and cysteine derivatives have been designed and synthesized based on the strategy of "medicinal chemical hybridization". Pharmacological evaluation indicated that the amide conjugates **3a**/**4a**/**17a** and thioester conjugates **6a**–**d** exhibited obvious protective effects on H_2O_2-induced human umbilical vein endothelial cells (HUVECs). Pretreated with these conjugates could increase glutathione (GSH) activity and decrease malondialdehyde (MDA) level. Further study on mechanism of compound **4a** revealed that it was related to its mitochondrial-protective effect and regulation of apoptosis-related proteins expression (Bax, p53, PARP, caspase-3, caspase-9 and Bcl-2). These results indicate that these Danshensu-cysteine analog conjugates possess significant cardiovascular-protective effects and merit further investigation.

1. Introduction

Danshensu, (*R*)-(+)-3-(3,4-dihydroxyphenyl)-2-hydroxypropanoic acid, is one of the main hydrosoluble active ingredients of *Radix Salviae Miltiorrhizae* (Danshen) which has been applied in clinical practice for centuries in Asia (Scheme 1). It has been widely reported on its pharmacological effects such as potent anti-oxidative, inhibiting platelet aggregation, anti-coagulant, anti-inflammatory, improving heart function effects and anti-tumor activities [1–3]. Moreover, Danshensu is one of the basic building blocks in many active components of culinary plants (sage, rosemary, mint, melissa, thyme, prunella, origan, sweet basil, etc.) mostly responsible for antiinfective, antiinflammatory, and antioxidative activity of these herbs, and therefore its scaffold is easily accepted by the human body and privileged for further exploring novel drug candidates among these activities [4,5]. In our previous work [4], a series of optically active Danshensu derivatives (**1**) were synthesized by asymmetric hydrogenation and the preliminary pharmacological studies showed that they exhibited significant activity for cardiovascular protection by blocking oxidative stress and apoptosis pathway. The *in vivo* test indicated that the key intermediate, 2-acetoxy caffeic acid (**2**) exhibited anti-myocardial ischemic effects featured by reducing infarction size and increasing the level of the intracellular enzymes detectable in serum. The phenolic esterified or etherified protection of Danshensu were not inclined to be oxidized, with high liposoluble and low toxic property (The median lethal dose of these Danshensu derivatives is 5 g/kg, assayed by the National Shanghai Center for New Drug Safety Evaluation and Research). These results prompted us to further design new chemical entities by combining Danshensu with other biologically active molecules under the guidance of "medicinal chemical hybridization" (MCH) which has been widely used to design polyvalent drug [6–13]. It can be carried out by joining two drugs through an appropriate linker or bond which is likely to be cleaved metabolically. The most obvious advantages are potentially increased affinity and improved predictable pharmacokinetic profile, because single product is capable of working at two separate pharmacological sites of action directly or following metabolism. Such examples abound in recent years [6–13].

Recently, ample publications have probed hydrogen sulfide (H_2S) which has been proposed as the third gasotransmitter besides NO and CO [14–19]. It has been confirmed that endogenous H_2S possesses many important physiological functions in the regulation of vasodilation and vascular reconstruction in recent years [20–23]. Its cardiovascular bioactivities include relaxing vascular smooth muscle, inhibiting the proliferation of vascular smooth muscle cells

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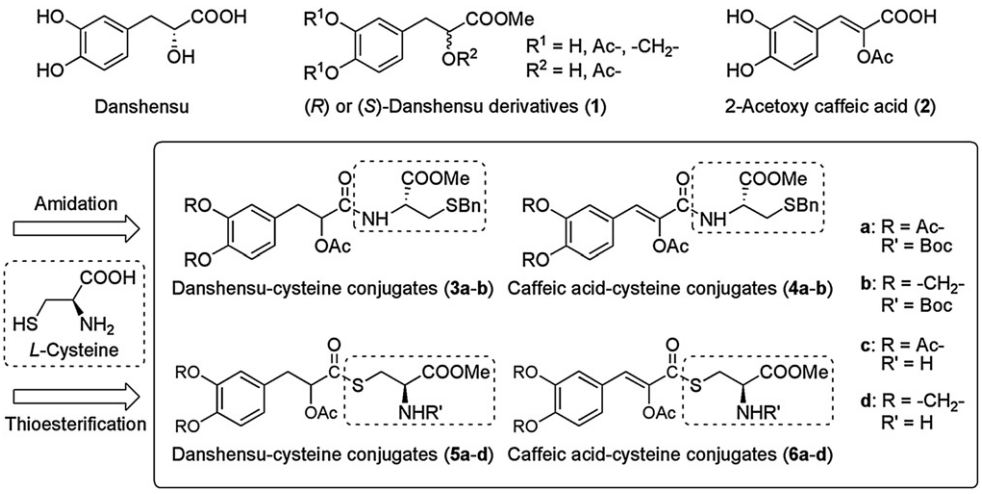


Scheme 1. The structures of Danshensu, L-cysteine and their conjugated derivatives.

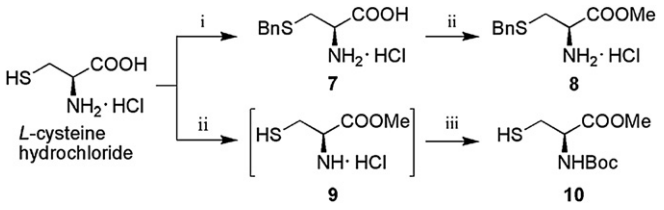
and preventing the generation of transient hypertension. Endogenous H_2S can also improve the ischemia-reperfusion injury by increasing the activity of antioxidant enzymes and direct removing part of the radical [21,22]. Endogenous H_2S is produced *in vivo* from cysteine, methionine and other organosulfur compounds in *Allium* species catalyzed by cystathionine-γ-lyase (CSE) and cystathionine-β-synthetase (CBS), two key enzymes of the *trans*-sulfuration pathway in the CNS and cardiovascular system, which leads to inactivation of HMG-CoA reductase and accordingly inhibits the biosynthesis of cholesterol [24].

Based on the MCH strategy, a series of novel amide conjugates of Danshensu-cysteine derivatives (**3a**–**b**) and 2-acetoxy caffeic acid-cysteine derivatives (**4a**–**b**) as well as the thioester conjugates (**5a**–**d**, **6a**–**d**) were designed via amidation and thioesterification to expect to enhance bioactivity through synergic effect (Scheme 1). In the present study, we reported the synthesis of these target conjugates, their pharmacological activities and the possible mechanism.

2. Chemistry

L-cysteine derivatives **8** and **10** with amino and mercapto group exposed respectively were prepared according to the general procedures outlined in Scheme 2. L-cysteine reacted with benzyl bromide in the presence of NaOH/EtOH solution to provided compound **7**, which was esterified by MeOH to obtain product **8** in 78% total yield. Compound **10** was attained via esterification of carboxyl group followed by protection of amino-group in **9** by *tert*-butoxycarboxylic anhydride in relatively low yield of 45% for two steps due to the unavoidable formation of its bisulfide even under N_2 atmosphere.

The designed conjugates were synthesized as outlined in Scheme 3 via the key intermediates **14a**–**b**, which could be facilely attained by the condensation of aldehydes **11a**–**b** with *N*-acetylglycine followed by hydrolysis in hydrochloric acid [4]. To prepare the racemic Danshensu derivatives **15a**–**b**, compounds **14a**–**b** were subjected to hydrogenation in the presence of catalytic amount of 10% Pd–C under the pressure of 15 atm and 1 atm respectively. Besides the normal products **15a**–**b** isolated in 68% and 30% yields respectively, the overreductive products **16a**–**b** were simultaneously obtained in yields of 15% and 59%, respectively. To investigate the effects of 2,3-double bond, 2-acetoxy group and phenolic protective groups on the bioactivity, six carboxylic acids **14**–**16** were condensed with L-cysteine derivative **8** to afford amide conjugates **4a**–**b**, **3a**–**b** and **17a**–**b** in good yields respectively. Similarly, the thioesterification between **14**–**16** and **10** were carried out in the presence of bis(2-oxo-3-oxazolidinyl) phosphonic chloride (BOP-Cl) and triethylamine (TEA) to afford thioester conjugates **6a**–**b**, **5a**–**b** and **18a**–**b** in 79–92% yields respectively. In order to get rid of the effect of the bulky Boc and release the amino groups, the deprotection were subsequently performed in the presence of TFA at 10 °C to give the corresponding conjugates **6c**–**d**, **5c**–**d** and **18c**–**d** in excellent yields [25].



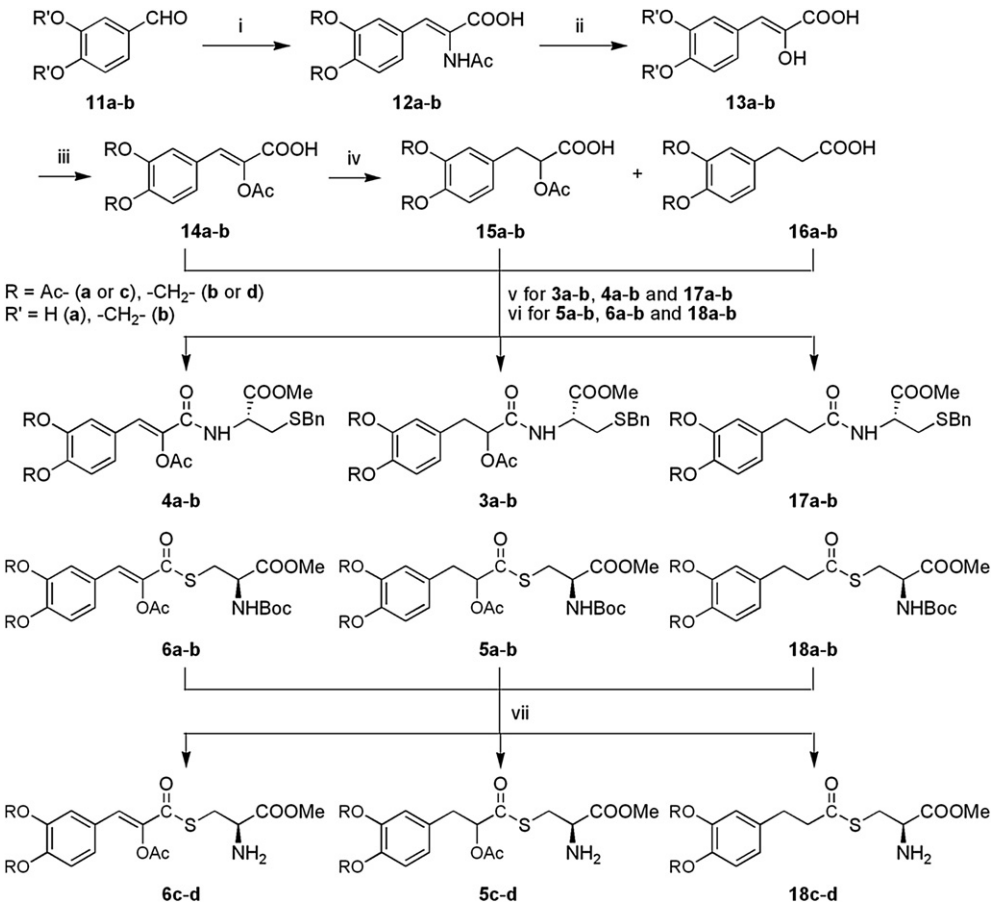
Scheme 2. The synthesis of L-cysteine derivatives **8** and **10**. Reaction conditions: (i) BnBr, NaOH/EtOH, rt, 1 h, 92%; (ii) MeOH, $SOCl_2$, rt, 6 h, 85% (**8**). (iii) Boc_2O, TEA, CH_2Cl_2 under N_2 atmosphere, 6 h, 45% yield for two steps.

3. Pharmacological assay results

3.1. Protective effects of Danshensu-cysteine analog conjugates on H_2O_2-induced HUVECs

Effects of these synthesized conjugates on the viability of H_2O_2-induced HUVECs were investigated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) analysis [26]. *N*-acetyl-L-cysteine (NAC) was served as positive control in the experiments. Cells were incubated with indicated concentrations of compounds for 4 h before exposure to 200 μmol/L H_2O_2 for 12 h. As shown in Fig. 1, 200 μM H_2O_2 treatment decreased cell viability to around 45%, but pre-treatment with **3a**/**4a**/**17a**, **6a**–**d**, **5d** and positive control NAC (5 mmol/L) prevented cell death, restoring cell viability ($^*P < 0.05$ vs. vehicle group).

As the activity of Boc deprotected conjugates are basically consistent with their precursors, compounds **3a**/**4a**/**17a** and **6a**–**b** were chosen for further investigation. The protective effects of these chosen Danshensu-cysteine analog conjugates were further evaluated by lactate dehydrogenase (LDH) assay, another indicator of cell injury [26]. Compounds **3a**/**4a**/**17a**, **6a**–**b** at the same concentration of 50 μmol/L and positive control NAC (5 mmol/L) were all effective in attenuating H_2O_2-induced increase in LDH release (Fig. 2A). As shown in Fig. 2B, compound **4a** could



Scheme 3. The synthesis of target conjugates. Reaction conditions: (i) *N*-acetylglycine, Ac_2O/NaOAc, 120 °C, 2–3 h, then poured into H_2O, yield: 75% (**12a**), 65% (**12b**); (ii) 9% HCl, 100 °C, 3–4 h, yield: 87% (**13a**), 96% (**13b**); (iii) Ac_2O/NaOAc, rt, 6 h, yield: 90% (**14a**), 92% (**14b**); (iv) H_2 (15 atm for **a** and 1 atm for **b**), 10% Pd–C, rt, 24 h, yield: 68% (**15a**), 15% (**16a**), 30% (**15b**), 59% (**16b**); (v) **8** (1.1 eq.), EDC, HOBt, DIPEA, rt, 2 h, yield: 78% (**3a**), 72% (**3b**), 80% (**4a**), 82% (**4b**), 75% (**17a**), 76% (**17b**); (vi) **10** (1.2 eq.), BOP-Cl, TEA, rt, 2–12 h, yield: 92% (**5a**), 87% (**5b**), 86% (**6a**), 89% (**6b**), 82% (**18a**), 79% (**18b**); (vii) TFA (2 eq.), CH_2Cl_2, 10 °C, 6 h, yield: 85% (**5c**), 89% (**5d**), 92% (**6c**), 94% (**6d**), 91% (**18c**), 88% (**18d**).

significantly decrease LDH leakage in a dose-dependent manner. The results were consistent with that determined by MTT assay. Therefore, the protective effect of these compounds on HUVECs against H_2O_2-induced injury was further evaluated.

3.2. Effects of compounds *3a*/*4a*/*17a* and *6a–b* on MDA and GSH in H_2O_2-induced HUVECs

To determine the antioxidant activity of compounds **3a**/**4a**/**17a** and **6a–b**, malondialdehyde (MDA), a marker of oxidant-mediated lipid peroxidation in cells and glutathione (GSH), an important antioxidant in cells, were quantified after H_2O_2-incubation. Treatment of HUVECs with 200 μmol/L of H_2O_2 for 12 h caused the increase in the level of MDA and the decrease in the activity of GSH. However, pre-incubation with compounds **3a**/**4a**/**17a**, **6a–b** at 50 μmol/L and positive control NAC (5 mmol/L) significantly inhibited the increase of MDA level and the loss of GSH activity (Fig. 3A and B) and compound **4a** manifested this antioxidant activity in a dose-dependent manner as indicated in Fig. 3C and D.

3.3. Compound *4a* abolished H_2O_2-induced mitochondrial membrane potential reduction in HUVECs

Mitochondrial membrane potential ($\Delta\psi_m$), tested by JC-1 assay, is an important parameter reflecting the function of mitochondria. JC-1 exhibits potential-dependent accumulation in mitochondria which is manifested by a fluorescence emission shift from green to red. As illustrated in Fig. 4, pre-treatment with compound **4a** dose-dependently restored the mitochondrial potential that decreased due to H_2O_2 exposure which was shown as decreased red fluorescence, indicating that compound **4a** possesses mitochondrial-protective effect on H_2O_2-induced HUVECs.

3.4. Compound *4a* attenuated H_2O_2-induced apoptosis in HUVECs

Apoptosis is a programmed cell death which is characterized by specific structural changes that include cell shrinkage, nuclear condensation and DNA fragmentation [27]. In order to evaluate the anti-apoptotic activity of **4a**, Hoechst staining was carried out to observe the morphological changes in H_2O_2-induced HUVECs. As shown in Fig. 5A, administration of compound **4a** (5, 50, 100 μmol/L) resulted in less nuclei-shrunk and nuclear condensation dose-dependently compared with the H_2O_2-induced group.

To gain insight into anti-apoptotic effects of compound **4a** in H_2O_2-induced HUVECs quantitatively, a display of PI versus Annexin V-FITC fluorescence was measured by flow cytometric analysis. Pre-incubation with compound **4a** (5, 50, 100 μmol/L) for 4 h prior to H_2O_2 exposure inhibited both late and total apoptosis dose-dependently and the values of total apoptosis were decreased to 20.1 ± 1.9%, 9.0 ± 0.7% and 5.4 ± 1.8%, respectively (Fig. 5B).

3.5. Effects of compound *4a* on the levels of apoptosis-related proteins

To further elucidate the mechanism of anti-apoptotic activity of compound **4a**, expression levels of apoptosis-related proteins were examined at protein level.

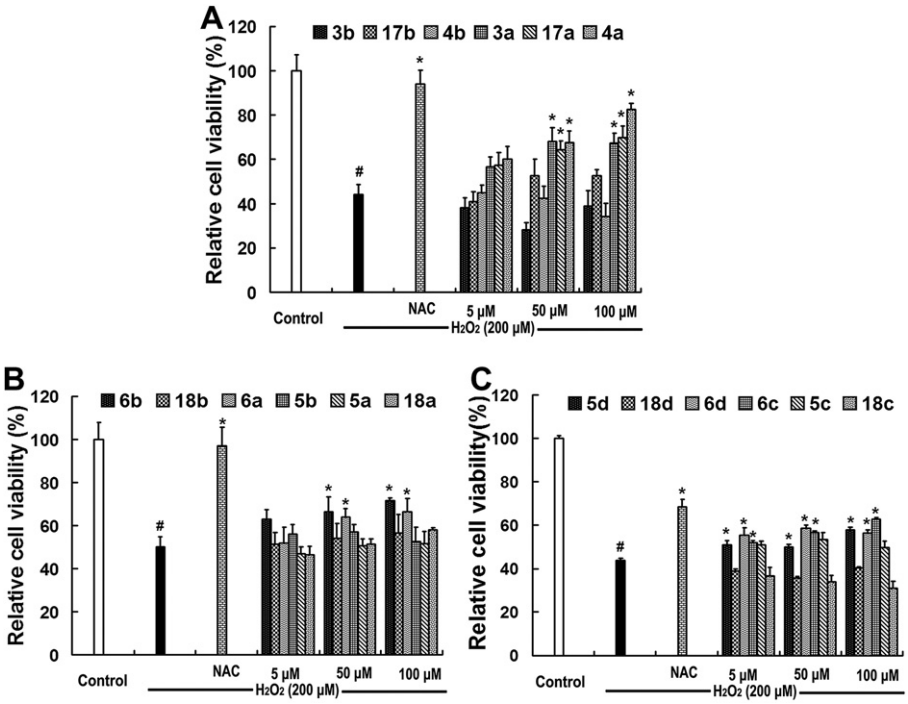


Fig. 1. (A) Effects of conjugates **3a**–**b**/**4a**–**b**/**17a**–**b** on cell viability in H_2O_2-induced HUVECs. (B) Effects of conjugates **5a**–**b**/**6a**–**b**/**18a**–**b** on cell viability in H_2O_2-induced HUVECs. (C) Effects of conjugates **5c**–**d**/**6c**–**d**/**18c**–**d** on cell viability in H_2O_2-induced HUVECs. The viability of control cell is presumed to be 100%. HUVECs were pre-incubated with different concentrations of danshensu-cysteine conjugates (5, 50, 100 μmol/L) and NAC (5 mmol/L) for 4 h and then exposed to H_2O_2 for 12 h. **NAC** denotes *N*-acetyl-L-cysteine. Values are expressed as means ± S.D. from six individual samples. $^{*}P < 0.05$ versus vehicle group; $^{\#}P < 0.05$ versus control group.

Western blot analysis revealed that the levels of pro-apoptotic proteins Bax, p53 and poly (ADP-ribose) polymerase (PARP) in H_2O_2-induced HUVECs declined significantly, whereas the level of anti-apoptotic protein Bcl-2 markedly increased, all in a dose-dependent manner, after treatment by compound **4a** compared with H_2O_2-induced group ($P < 0.05$) (Fig. 6). As shown in Fig. 7, the amount of pro-caspase-3 and pro-caspase-9 in H_2O_2-induced HUVECs were markedly decreased, which is a sign of caspase activation that lead to apoptosis. However, compound **4a** pretreatment attenuated the H_2O_2-induced caspase-3 and caspase-9 activation dose-dependently ($P < 0.05$). All of these results indicate that compound **4a** inhibits apoptosis markedly in H_2O_2-induced HUVECs.

4. Discussion

Enhanced oxidative damage after various stimuli has been confirmed to be an initial event in the development of cardiovascular diseases. Human umbilical vein endothelial cells (HUVECs) are commonly accepted as a tool in the study of the mechanisms involved in the pathogenesis of cardiovascular diseases, and hydrogen peroxide (H_2O_2) is extensively referred to as one of the major oxidative stimuli in antioxidative studies [28–30]. In the present study, a series of Danshensu-cysteine analog conjugates were synthesized and their pharmacological activities as well as possible protective mechanism were investigated on H_2O_2-induced HUVECs.

MTT results showed that the amide conjugates **3a**/**4a**/**17a** with phenolic acetyl protective groups increased cell viability significantly after H_2O_2 treatment, indicating that both Danshensu and caffeic acid were effective scaffolds in this type of conjugates. However, the amidates **3b**/**4b**/**17b** with the methylene protective groups were inert, which may be attributed to the fact that their methylene phenolic protective groups were hard to cleave to release hydroxyls compared with acetyl in **3a**/**4a**/**17a**. All thioester conjugates **6a**–**d** with caffeic acid scaffold were demonstrated

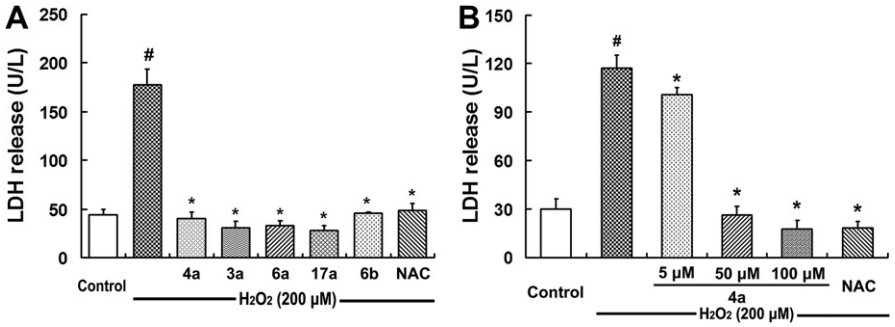


Fig. 2. (A) Effects of **3a**/**4a**/**17a** and **6a**–**b** on LDH release in H_2O_2-induced HUVECs. HUVECs were pre-incubated with 50 μmol/L **3a**/**4a**/**17a**, **6a**–**b** and 5 mmol/L NAC for 4 h and then exposed to H_2O_2 for 12 h. (B) Effect of **4a** on LDH release in H_2O_2-induced HUVECs. HUVECs were pre-incubated with 5, 50, 100 μmol/L **4a** and 5 mmol/L NAC for 4 h and then exposed to H_2O_2 for 12 h. **NAC** denotes *N*-acetyl-L-cysteine. Values are expressed as means ± S.D. from three individual samples. $^{*}P < 0.05$ versus vehicle group; $^{\#}P < 0.05$ versus control group.

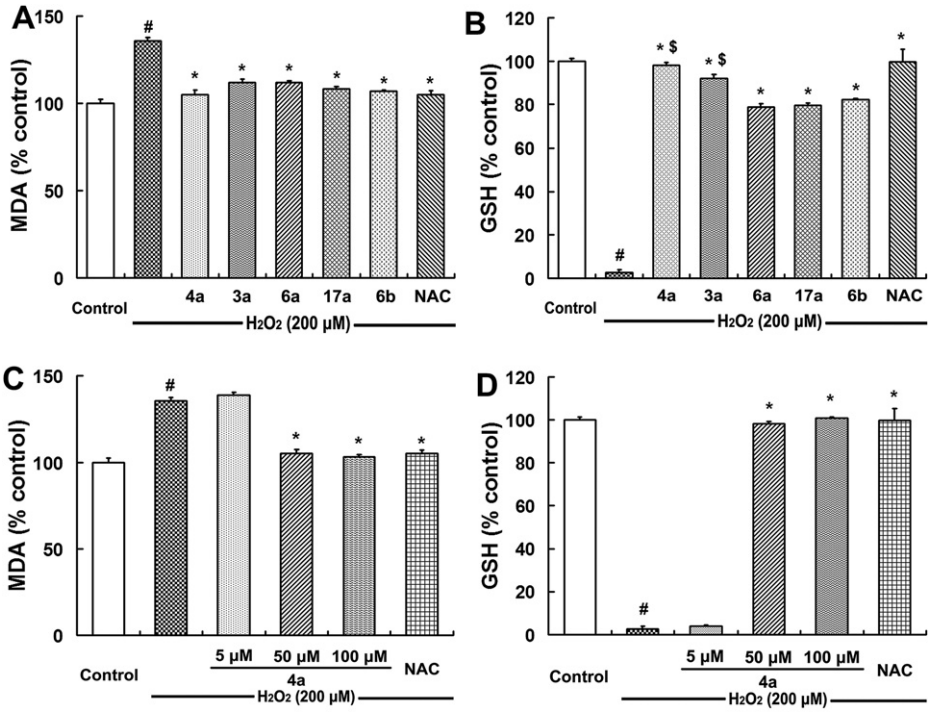


Fig. 3. (A) Effects of **3a**/**4a**/**17a** and **6a**–**b** on MDA level in H_2O_2-induced HUVECs. (B) Effects of **3a**/**4a**/**17a** and **6a**–**b** on total GSH level in H_2O_2-induced HUVECs. HUVECs were pre-incubated with 50 μmol/L **3a**/**4a**/**17a**, **6a**–**b** and 5 mmol/L NAC for 4 h and then exposed to H_2O_2 for 12 h. (C) Effect of **4a** on MDA level in H_2O_2-induced HUVECs. (D) Effect of **4a** on total GSH level in H_2O_2-induced HUVECs. HUVECs were pre-incubated with 5, 50, 100 μmol/L **4a** and 5 mmol/L NAC for 4 h and then exposed to H_2O_2 for 12 h. **NAC** denotes *N*-acetyl-L-cysteine. Values are expressed as means ± S.D. from three individual samples. $^{*}P < 0.05$ versus vehicle group; $^{\#}P < 0.05$ versus control group; $^{\$}P < 0.05$ versus **6a**, **6b**, **17a**–treated groups, respectively.

bioactive albeit most thioesters (**5a**–**c**/**18a**–**d**) were inactive, suggesting that the carbon–carbon double bond was the crucial pharmacophore in the thioester conjugates.

Compounds **3a**/**4a**/**17a** and **6a**–**b** all decreased LDH leakage after H_2O_2 treatment and manifested potent antioxidant activity featured by decreased formation of MDA and increased GSH activity in H_2O_2-induced HUVECs. At the same concentration of 50 μmol/L, the GSH activity of cells treated by the amide conjugates **3a**/**4a** was higher than that of cells treated by the thioester conjugates **6a**–**b**, illuminating that the bioactivity of amide conjugates is more potent than those conjugated by thioester bond. Besides, the fact that **3a**/**4a** exhibited higher activities than **17a** in GSH assay showed that 2-acetoxy substitution was favorable to anti-oxidant activity. However, no significant difference of GSH activity was exhibited between **4a** and **3a** treated cells, indicating that the double bond was not the crucial pharmacophore in the amide conjugates.

Morphological analysis via Hoechst staining showed that **4a** lessened the strong staining and nuclear shrunk after H_2O_2 exposure. Meanwhile, the flow cytometric evaluation gave similar results that **4a** dose-dependently inhibited both late and total apoptosis in H_2O_2-induced HUVECs. These results suggested that **4a** possessed potent anti-apoptotic activity.

Mitochondria are complex cell organelles which are essential in the maintenance of cell survival. Mitochondrial dysfunction is a prominent feature of cell apoptosis, which is marked by a loss of mitochondrial membrane potential. It has been revealed that the intrinsic program of apoptosis was mainly triggered by the mitochondrial pathway [31]. Therefore, to further elucidate the protective mechanism of **4a**, its inhibitory effect on the loss of mitochondrial membrane potential was determined. As expected, pretreatment of **4a** greatly prevented HUVECs from H_2O_2-induced reduction of mitochondrial membrane potential, indicating that anti-apoptotic activity of **4a** was related to its protection of mitochondria.

The members of Bcl-2 family, a series of anti- or pro-apoptotic regulators, are critical for the regulation of apoptosis. Bcl-2 protein is known to promote cell survival as well as to suppress cell death, whereas Bax is a pro-apoptotic protein that promotes or accelerates cell death [32]. P53 is a tumor suppressor protein which can lead to apoptosis. The results showed that **4a** up-regulated the level of Bcl-2 markedly and down-regulated the levels of Bax and p53. The caspase family is another key regulator of apoptotic signaling pathway, which is categorized into initiator (caspase-8, -9) and executioner (caspase-3, -6, -7) [33]. Damaged mitochondria will activate upstream inactive proteases (caspase-9) to trigger a cascade reaction which finally turns downstream executive proteins such as caspase-3 into active forms. Active executive protein caspase-3 can further cleave downstream substrates involved in apoptotic process, such as PARP, resulting in cell apoptosis [28,34,35]. In the current study, H_2O_2-induced activation of caspase-9 and caspase-3, increased p53 and Bax expression, as well as the cleavage of PARP were all inhibited by pretreated **4a** significantly. Therefore, the mechanism by which **4a** suppressed cell apoptosis in H_2O_2-induced HUVECs was possibly through the regulation in the intrinsic mitochondria-mediated pathway such as Bcl-2 and caspase family-related signals.

5. Conclusion

The synthesis and pharmacological evaluation of a series of novel amide and thioester conjugates between Danshensu and cysteine derivatives have been reported and **3a**/**4a**/**17a**/**6a**–**d** demonstrated significant protective effect on H_2O_2-induced

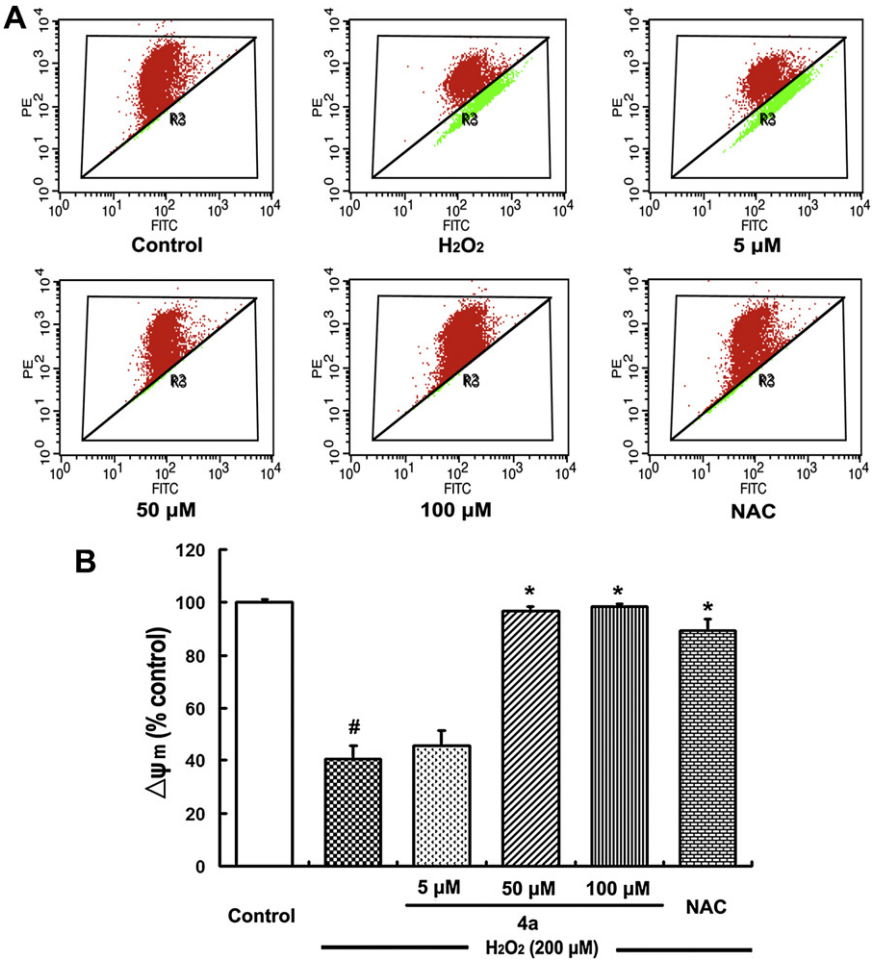


Fig. 4. Effect of **4a** on loss of mitochondrial membrane potential in H_2O_2-induced HUVECs. **NAC** denotes *N*-acetyl-L-cysteine. Values are expressed as means ± S.D. from three individual samples. $^{*}P < 0.05$ versus vehicle group; $^{\#}P < 0.05$ versus control group.

HUVECs. Pharmacological evaluation has shown that these conjugates exhibit well-ordered structure–activity relationship. For amide conjugates **3a**/**4a**/**17a**, the phenolic acetyl protective groups and 2-acetoxy substitution are favorable to their activity. For thioester conjugates **6a**–**d**, the scaffold of caffeic acid was more privileged than saturated analogs. The protective mechanism of these conjugates may be related to their anti-oxidative and anti-apoptotic properties. Further study are in progress as these promising results demonstrate that these Danshensu-cysteine analog conjugates merit further investigation as potential cardiovascular-protective agents.

6. Experimental

Starting materials and reagents were obtained from commercial suppliers and were used without purification. Melting points were determined in open capillary tubes on X-4 apparatus and were uncorrected. Nuclear magnetic resonance spectra were recorded on a Brucker-DPX 400 MHz spectrometer. Mass spectral data was collected on Shimadzu LCMS-2010EV, Agilent 5973N MSD and Agilent LC/MSD analytical mass spectrometer. HRMS data were determined on Kratos Concept 1H and Bruker Daltonics or IonSpec 4.7 T FTMS instrument. H_2O_2, *N*-acetyl-L-cysteine (NAC), 3-(4, 5-dimetrylthiazol)-2, 5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO) were purchased from Sigma Chemical (St. Louis, MO, USA). Anti-Bax, anti-Bcl-2, anti-caspase-9 and anti-β-actin were purchased from Proteintech Group, Inc. (IL, USA); anti-p53 and anti-caspase-3 were obtained from Cell Signaling Technology, Inc. (MA, USA); anti-(ADP-ribose)polymerase (PARP) was purchased from Epitomics, Inc. (CA, USA). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were obtained from Gibco (Gibco-BRL, Paisley, UK). The kits for lactate dehydrogenase (LDH), malondialdehyde (MDA), glutathione (GSH) were purchased from Jiancheng Bioengineering Institute (Nanjing, China). The kits for JC-1 and Hoechst 33258 staining were obtained from Beyotime Bioengineering Institute (Lianyungang, China). The kit for AnnexinV-FITC/PI was purchased from BD Biosciences (CA, USA). All compounds (except for positive control NAC) solution in dimethyl sulfoxide (DMSO) was freshly prepared. Final DMSO concentration in media did not exceed 0.05%.

6.1. (R)-Methyl 2-amino-3-(benzylthio)propanoate hydrochlorid (*8*)

To a rapidly stirring solution of 2*N* sodium hydroxide (15 mL) and ethanol (35 mL) was added L-cysteine hydrochloride (1.3 g, 8.2 mmol) and benzyl bromide (0.98 mL, 8.2 mmol). The reaction mixture was neutralized after 1 h to pH 6–7 by careful addition of concentrated hydrochloric acid. The precipitate was filtered and washed successively with water, ethanol and ether to give *S*-benzylcysteine (**7**) as a white powder (1.87 g, 92%), mp 210–212 °C (lit [36].: 215–216 °C). To a solution of compound **7** (1 g, 5 mmol) in

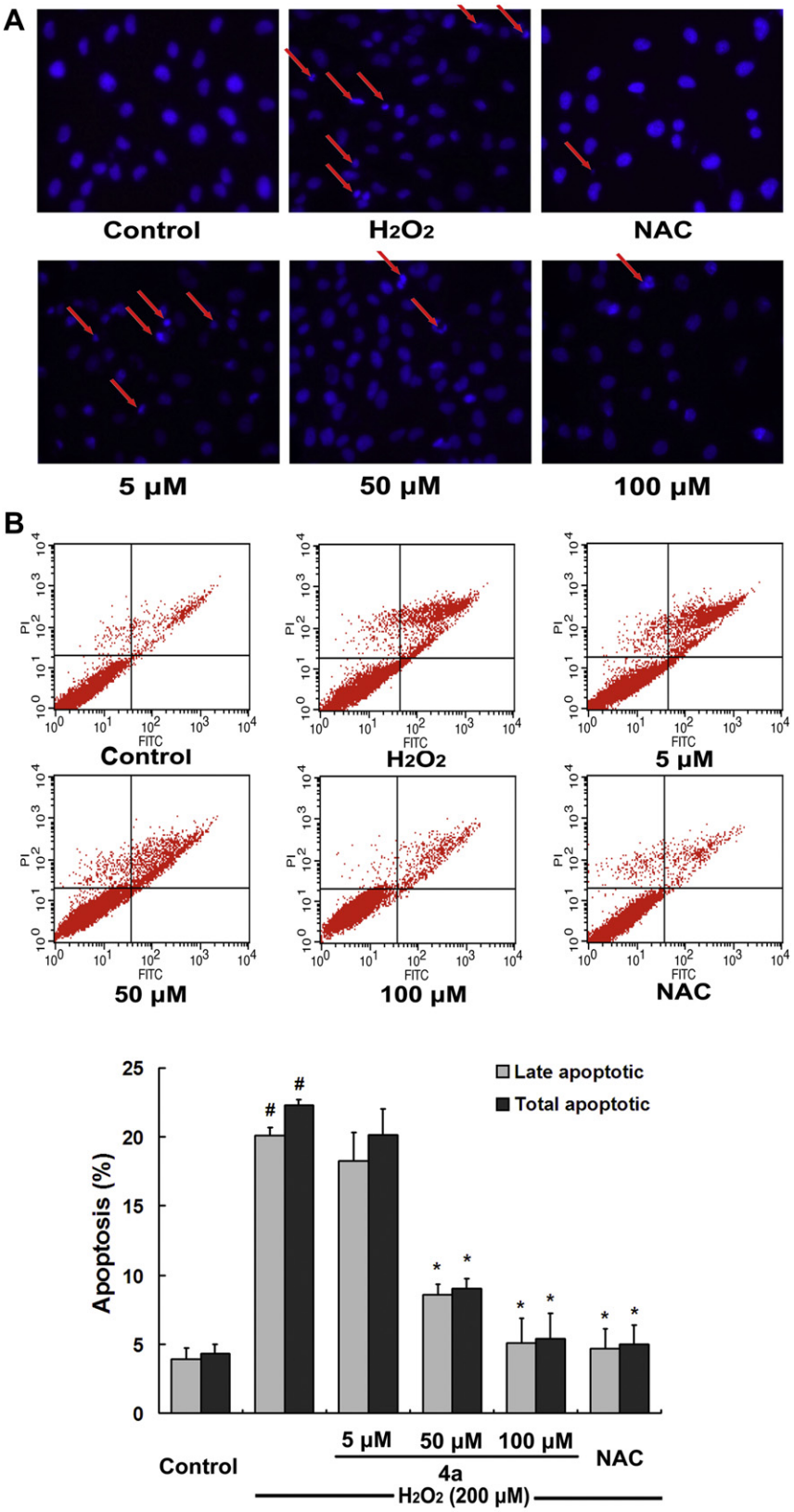


Fig. 5. (A) Effect of **4a** on morphological features in H_2O_2-induced HUVECs. Fluorescence photomicrographs of cells stained with Hoechst 33258 (400 ×). Each photograph is a representative of three independent observations. (B) Anti-apoptotic effect of **4a** in H_2O_2-induced HUVECs determined by AnnexinV-FITC/PI. **NAC** denotes *N*-acetyl-L-cysteine. Values are expressed as means ± S.D. from three individual samples. $^{*}P < 0.05$ versus vehicle group; $^{\#}P < 0.05$ versus control group.

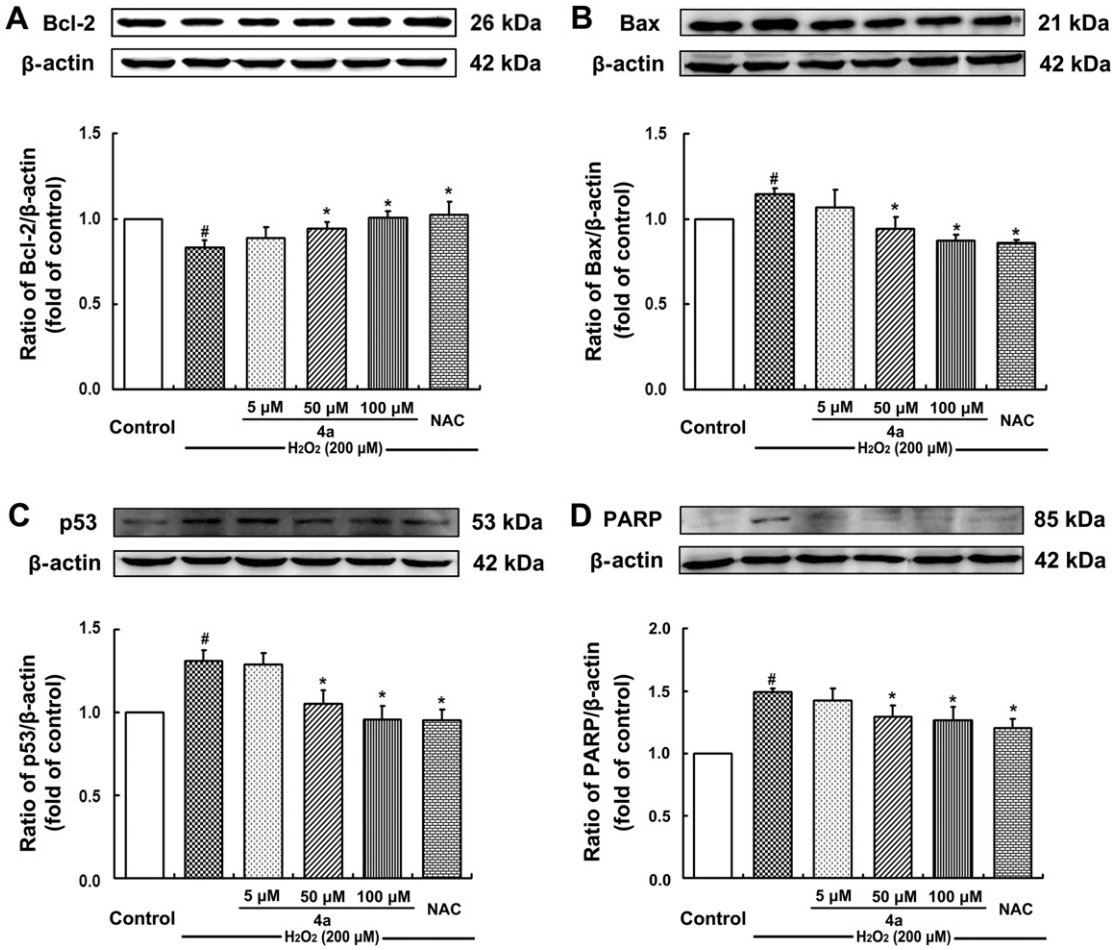


Fig. 6. (A) Effect of **4a** on the protein expression of Bcl-2 in H_2O_2-induced HUVECs. (B) Effect of **4a** on the protein expression of Bax in H_2O_2-induced HUVECs. (C) Effect of **4a** on the protein expression of p53 in H_2O_2-induced HUVECs. (D) Effect of **4a** on the protein expression of PARP in H_2O_2-induced HUVECs. **NAC** denotes *N*-acetyl-L-cysteine. Values are expressed as means ± S.D. from three individual samples. $^{*}P < 0.05$ versus vehicle group; $^{\#}P < 0.05$ versus control group.

methanol (30 mL) at 0 °C was dropped $SOCl_2$ (1.5 mL, 7 mmol) and then the mixture was stirred at room temperature for 6 h. Methanol was evaporated to give **8** as a white solid (1.3 g, 85%), mp 148–150 °C (lit [37].: 160–162 °C; lit. [38].: 151–152 °C). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.23–7.32 (m, 5H, Ar–H), 3.73 (s, 2H, $-CH_2Ph$), 3.72 (s, 3H, $-COOCH_3$), 3.58–3.61 (m, 1H, H-2), 2.80–2.85 (m, 1H, H-3), 2.64–2.69 (m, 1H, H-3).

*6.2. (R)-Methyl 2-(tert-butoxycarbonylamino)-3-mercaptopropanoate (**10**)*

To a solution of L-cysteine hydrochloride (2 g, 12.6 mmol) in methanol (40 mL) at 0 °C was dropped $SOCl_2$ (1.5 mL, 7 mmol) and then the mixture was reacted at room temperature for 24 h. Methanol was evaporated to give **9** as a colorless oil which was

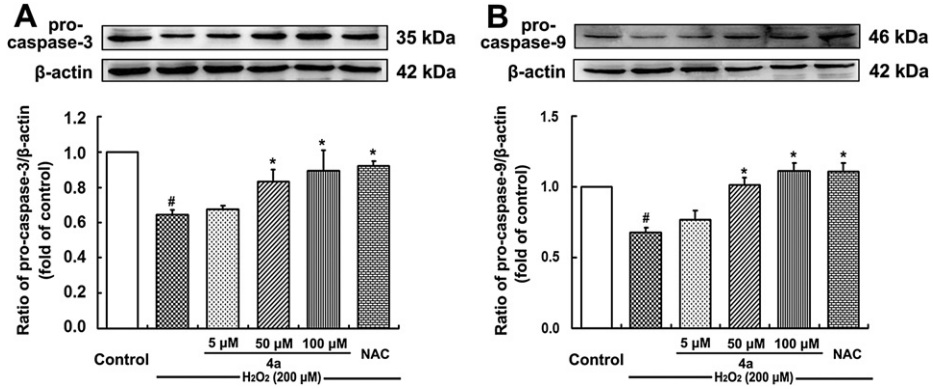


Fig. 7. (A) Effect of **4a** on the protein expression of pro-caspase-3 in H_2O_2-induced HUVECs. (B) Effect of **4a** on the protein expression of pro-caspase-9 in H_2O_2-induced HUVECs. **NAC** denotes *N*-acetyl-L-cysteine. Values are expressed as means ± S.D. from three individual samples. $^{*}P < 0.05$ versus vehicle group; $^{\#}P < 0.05$ versus control group.

directly used in the next process. To a solution of **9** and triethylamine (2.1 mL, 15 mmol) in CH_2Cl_2 (30 mL) was added Boc_2O and reacted under N_2 atmosphere for 6 h. The solid was filtered out and the filtrate was purified by silica gel column chromatography (eluent: PE-EtOAc, 10:1–3:1) providing **10** as a colorless oil (1.33 g, 45% for two steps). ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.41 (br s, 1H, –NH–), 4.58–4.60 (m, 1H, H-2), 3.76 (s, 3H, $-COOCH_3$), 2.93–2.96 (m, 2H, H-3), 1.78 (s, 1H, –SH), 1.46 (s, 9H, $-OC(CH_3)_3$). The ^{1}H NMR data are consistent with those reported in literature [39].

6.3. 2-Acetoxy-3-(3,4-diacetoxyphenyl)propanoic acid (15a) and 3-(3,4-diacetoxyphenyl)propanoic acid (**16a**)

(*Z*)-2-Acetoxy-3-(3,4-diacetoxyphenyl)acrylic acid (**14a**) and (*Z*)-2-acetoxy-3-(3,4-methylenedioxyphenyl)acrylic acid (**14b**) were prepared by using aldehydes **11a–b** as the starting materials according to the literature method [4].

A mixture of **14a** (1 g, 3.1 mmol), 10% Pd–C (200 mg) and MeOH (9 mL) was hydrogenated at 15 atm for 24 h. The mixture was filtered and concentrated, the residue was purified by silica gel column chromatography (eluent: PE-EtOAc, 20:1–5:1), providing **15a** (0.68 g, 68%) and **16a** (148 mg, 15%) as an oil and a white solid respectively. **15a**: ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.09–7.12 (m, 3H, Ar–H), 5.21 (br s, 1H, –COOH), 3.12–3.18 (m, 2H, H-3), 2.27 (s, 6H, Ar–$OCOCH_3$), 2.17 (s, 3H, CH_3COO-2). **16a**: mp 75–78 °C (lit [40].: 79–80 °C), ^{1}H NMR ($CDCl_3$) δ 7.10 (s, 2H, Ar–H), 7.04 (s, 1H, Ar–H), 2.95 (t, J = 7.6 Hz, 2H, H-2), 2.68 (t, J = 7.6 Hz, 2H, H-3), 2.28 (s, 6H, $-OCOCH_3$).

6.4. 2-Acetoxy-3-(3,4-methylenedioxyphenyl)propanoic acid (**15b**) and 3-(3,4-methylenedioxyphenyl)propanoic acid (**16b**)

A mixture of **14b** (525 mg, 3.1 mmol), 10% Pd–C (103 mg) and MeOH (9 mL) was hydrogenated at atmospheric pressure for 24 h. The mixture was filtered and concentrated, the residue was purified by silica gel column chromatography (eluent: CH_2Cl_2–CH_3OH, 8:1), providing **15b** (130 mg, 30%) and **16b** (230 mg, 59%) as pale yellow solids respectively. **15b** was purified by crystallization from acetic acid to give **15b** as white solid: mp 150–152 °C (lit [4].: 151–153 °C). ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.68–6.75 (m, 3H, Ar–H), 5.94 (s, 2H, $-OCH_2O-$), 5.17–5.21 (m, 1H, H-2), 3.01–3.16 (m, 2H, H-3), 2.12 (s, 3H, $-OCOCH_3$). **16b**: mp 80–82 °C (lit [41].: 87–88 °C), ^{1}H NMR ($CDCl_3$) δ 6.66–6.75 (m, 3H, Ar–H), 5.93 (s, 2H, $-OCH_2O-$), 2.89 (t, J = 7.2 Hz, 2H, H-3), 2.64 (t, J = 7.2 Hz, 2H, H-2).

6.5. General procedure for the amide conjugates **3a–b**, **4a–b** and **17a–b**

To a solution of carboxylic acid **15a–b**, **14a–b** or **16a–b** (0.5 mmol) in CH_2Cl_2 (8 mL) was added 1-hydroxybenzotriazole (HOBt, 82 mg, 0.6 mmol) and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDCI, 116 mg, 0.6 mmol). The mixture was stirred at room temperature for 15 min, then methyl *S*-benzyl-L-cysteine hydrochloride (**8**) (244 mg, 0.55 mmol) followed by diisopropylethylamine (0.1 mL, 0.6 mmol) were added. The solution was kept at room temperature for 2 h and then purified by silica-gel column chromatography to give the corresponding amide conjugates **3a–b**, **4a–b** and **17a–b**.

6.5.1. N-((R)-3-Benzylthio-1-methoxy-1-oxo-2-propanyl)-2-acetoxy-3-(3,4-diacetoxyphenyl)propanamide (**3a**)

Eluent: PE-EtOAc, 1:1. Sticky oil, yield 78%. $[\alpha]_D^{25}$ +0.2 (c 1.0, $CHCl_3$). UV λ_{max} (nm) (log ε) (MeOH): 203 (4.61). IR (neat), ν_{max} (cm^{-1}): 1771, 1748, 1679, 1507, 1437, 1372, 1213. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.24–7.33 (m, 5H, –Ph), 7.05–7.09 (m, 3H, Ar–H), 6.75 (t, J = 7.9 Hz, 1H, –N***H***CH–), 5.36–5.40 (m, 1H, $ArCH_2C$***H***–), 4.71–4.75 (m, 1H, –NHC***H***–), 3.74 (s, 3H, $-COOCH_3$), 3.65 (s, 2H, $-SCH_2Ph$), 3.11–3.21 (m, 2H, ArC***H***$_2$CH-), 2.78–2.87 (m, 2H, $-CH_2SBn$), 2.25–2.27 (m, 6H, 2 × Ar–$OCOCH_3$), 2.11, 2.13 (2s, 3H, CH_3COO-2). ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.8, 169.5, 168.8, 168.2, 168.1, 141.8, 141.0, 137.5, 134.5, 129.0, 128.9, 128.6, 127.6, 127.3, 124.7, 123.3, 73.7, 52.7, 51.2, 36.9, 36.4, 33.3, 33.0, 20.8, 20.6. ESI-MS *m*/*z* (%): 554.8 (M + Na^+, 100). HRMS calcd mass for $C_{26}H_{30}NO_9S$ [M + H^+] 532.1636, found 532.1633.

6.5.2. N-((R)-3-Benzylthio-1-methoxy-1-oxo-2-propanyl)-2-acetoxy-3-(3,4-methylenedioxyphenyl)propanamide (**3b**)

Eluent: PE-EtOAc, 2:1. White solid, yield 72%, mp 84–85 °C. $[\alpha]_D^{25}$ –27.5 (c 0.5, acetone). UV λ_{max} (nm) (log ε) (MeOH): 206 (4.49), 286 (3.56). IR (neat), ν_{max} (cm^{-1}): 3361, 2925, 1742, 1674, 1491, 1443, 1370, 1245, 1038, 931, 808, 704. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.24–7.35 (m, 5H, –Ph), 6.58–6.79 (m, 4H, –N***H***CH–, Ar–H), 5.87–5.92 (m, 2H, $-OCH_2O-$), 5.32–5.37 (m, 1H, $ArCH_2C$***H***–), 4.73–4.79 (m, 1H, –NHC***H***–), 3.74 (s, 3H, $-COOCH_3$), 3.64, 3.66 (s, s, 2H, $-SCH_2Ph$), 3.00–3.17 (m, 2H, ArC***H***$_2$CH–), 2.80–2.88 (m, 2H, $-CH_2SBn$), 2.11, 2.15 (2s, 3H, CH_3COO-2). ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.8, 169.4, 168.8, 147.6, 146.6, 137.4, 128.9, 128.6, 127.3, 122.7, 122.6, 109.9, 109.8, 108.2, 74.3, 52.7, 51.3, 37.3, 36.5, 36.4, 20.9. ESI-MS *m*/*z* (%): 460.2 (M + H^+, 100). HRMS (MALDI-DHB) calcd mass for $C_{23}H_{25}NO_7SNa$ [M + Na^+] 482.1244, found 482.1244.

6.5.3. N-((R)-3-Benzylthio-1-methoxy-1-oxo-2-propanyl)-2-acetoxy-3-(3,4-diacetoxyphenyl)propenamide (**4a**)

Eluent: PE-EtOAc, 1:1. White needles, yield 80%: mp 111–112 °C. $[\alpha]_D^{25}$ –29 (c 1.0, $CHCl_3$). UV λ_{max} (nm) (log ε) (MeOH): 202 (4.56), 289 (4.13), 320 (4.20). IR (KBr), ν_{max} (cm^{-1}): 1745, 1671, 1640, 1504, 1449, 1244, 1184, 1308. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.18–7.44 (m, 9H, Ar–H, -CH=), 6.84 (d, J = 7.3 Hz, 1H, –N***H***CH–), 4.84–4.86 (m, 1H, –NHC***H***–), 3.77 (s, 3H, $-COOCH_3$), 3.70 (s, 2H, $-SCH_2Ph$), 2.91–3.03 (m, 2H, $-SCH_2-$), 2.34 (s, 3H, $-OCOCH_3$), 2.30 (s, 6H, Ar–$OCOCH_3$). ^{13}C NMR (100 MHz, $CDCl_3$) δ 171.0, 167.9, 167.8, 162.0, 142.5, 142,1, 139.8, 137.5, 130.8, 128.9, 128.6, 127.9, 127.3, 123.7, 52.8, 51.9, 36.6, 33.2, 20.7. ESI-MS *m*/*z* (%): 530.0 (M + H^+, 100). HRMS calcd mass for $C_{26}H_{28}NO_9S$ [M + H^+] 530.1479, found 530.1467.

6.5.4. N-((R)-3-Benzylthio-1-methoxy-1-oxo-2-propanyl)-2-acetoxy-3-(3,4-methylenedioxyphenyl)propenamide (**4b**)

Eluent: PE-EtOAc, 1:1. White needles, yield 82%: mp 100–102 °C. $[\alpha]_D^{25}$ –30.0 (c 1.0, $CHCl_3$). UV λ_{max} (nm) (log ε) (MeOH): 205 (4.38), 289 (3.85), 320 (3.91). IR (neat), ν_{max} (cm^{-1}): 2958, 2925, 2855, 1766, 1638, 1493, 1187, 1036, 967. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.24–7.31 (m, 5H, –Ph), 7.16 (s, 1H, Ar–H), 7.08 (s, 1H, –CH=C–), 7.02 (d, J = 8.3 Hz, 1H, Ar–H), 6.77–6.84 (m, 2H, –N***H***CH–, Ar–H), 6.00 (s, 2H, $-OCH_2O-$), 4.84–4.86 (m, 1H, –NHC***H***–), 3.77 (s, 3H, $-COOCH_3$), 3.70 (s, 2H, $-SCH_2Ph$), 2.91–3.03 (m, 2H, $-SCH_2-$), 2.36 (s, 3H, $-OCOCH_3$). ^{13}C NMR (100 MHz, $CDCl_3$) δ 171.1, 167.9, 162.4, 148.7, 148.0, 137.6, 137.5, 128.9, 128.6, 127.3, 126.2, 125.5, 124.3, 108.8, 108.6, 101.5, 52.8, 51.9, 36.7, 33.3, 20.9. ESI-MS *m*/*z* (%): 458.0 (M + H^+, 100). HRMS calcd mass for $C_{23}H_{24}NO_7S$ [M + H^+] 458.1273, found 458.1286.

6.5.5. N-((R)-3-Benzylthio-1-methoxy-1-oxo-2-propanyl)-3-(3,4-diacetoxyphenyl)propanamide (**17a**)

Eluent: PE-EtOAc, 1:1. White solid, yield 75%: mp 86–88 °C. $[\alpha]_D^{25}$ +4.8 (c 1.0, $CHCl_3$). UV λ_{max} (nm) (log ε) (MeOH): 202 (4.56). IR (neat), ν_{max} (cm^{-1}): 3370, 2924, 1745, 1652, 1504, 1436, 1370, 1207, 1181, 1109, 1013, 897, 703. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.24–7.33 (m, 5H, –Ph), 7.06–7.08 (m, 2H, Ar–H), 7.04 (s, 1H, Ar–H), 6.08 (d, J = 7.4 Hz, 1H, –NH–), 4.77–4.78 (m, 1H, –NHC***H***–),

3.73 (s, 3H, −$COOCH_3$), 3.67 (s, 2H, −SCH_2Ph), 2.95 (t, J = 7.5 Hz, 2H, ArC***H***$_2$CH−), 2.84–2.87 (m, 2H, −CH_2SBn), 2.46–2.51 (m, 2H, $ArCH_2$C***H***−), 2.27 (s, 6H, −$OCOCH_3$). ^{13}C NMR (100 MHz, $CDCl_3$) δ 171.4, 171.2, 168.3, 168.2, 142.0, 140.5, 139.5, 137.8, 128.9, 128.7, 127.3, 126.5, 123.3, 52.6, 21.6, 37.5, 36.7, 33.5, 30.6, 20.6. ESI-MS *m*/*z* (%): 496.12 (M + Na^+, 100). HRMS calcd mass for $C_{24}H_{28}NO_7S$ [M + H^+] 474.1581, found 474.1574.

*6.5.6. N-((R)-3-Benzylthio-1-methoxy-1-oxo-2-propanyl)-3-(3,4-methylenedioxy phenyl)propanamide (**17b**)*

Eluent: PE-EtOAc, 2:1. White needles, yield 76%: mp 76–78 °C. $[\alpha]_D^{25}$ +7.6 (c 1.0, $CHCl_3$). UV λ_{max} (nm) (log ε) (MeOH): 204 (4.63), 286 (3.62). IR (neat), ν_{max} (cm^{-1}): 1744, 1654, 1491, 1443, 1246, 1039, 704. ^{1}HNMR (400 MHz, $CDCl_3$) δ 7.22–7.34 (m, 5H, −Ph), 6.64–6.72 (m, 3H, Ar−H), 6.06 (d, J = 6.3 Hz, 1H, −NH−), 5.89 (s, 2H, −OCH_2O−), 4.77–4.81 (m, 1H, −NHC***H***−), 3.73 (s, 3H, −$COOCH_3$), 3.65 (s, 2H, −SCH_2Ph), 2.81–2.89 (m, 4H, ArC***H***$_2$CH−, −CH_2SBn), 2.43–2.48 (m, 2H, $ArCH_2$C***H***−). ^{13}C NMR (100 MHz, $CDCl_3$) δ 171.7, 171.3, 147.7, 146.0, 137.7, 134.4, 128.9, 128.6, 127.3, 121.2, 108.8, 108.3, 100.8, 77.2, 52.6, 51.5, 38.3, 36.7, 33.5, 31.1. ESI-MS *m*/*z* (%): 402.1 (M + H^+, 100). HRMS calcd mass for $C_{21}H_{23}NO_5SNa$ [M + Na^+] 424.1195, found 424.1192.

*6.6. General procedure for the thioester conjugates **5a–b**, **6a–b** and **18a–b***

To a solution of carboxylic acid **15a–b**, **14a–b** or **16a–b** (1 mmol) in CH_2Cl_2 (10 mL) was added methyl *N*-(*tert*-butoxycarbonyl)-L-cysteine (**10**) (282 mg, 1.2 mmol). The mixture was stirred at 0 °C for 15 min, then triethylamine (0.5 mL, 4 mmol) followed by bis(2-oxo-3-oxazolidinyl)phosphonic chloride (BOP-Cl) (381 mg, 1.5 mmol) were added. The solution was kept at room temperature for 3 h. After the removal of the solvent under the reduced pressure, the residue was added EtOAc (30 mL) and washed with water (3 × 2 mL), brine (10 mL) and dried over anhydrous Na_2SO_4. After the solvent was removed under the reduced pressure, the residue was purified by silica-gel column chromatography to give the corresponding thioester conjugates **5a–b**, **6a–b** or **18a–b**.

*6.6.1. S-((R)-3-methoxy-3-oxo-2-(tert-butoxycarbonylamino)propyl) 2-acetoxy-3-(3,4-diacetoxyphenyl)propanethioate (**5a**)*

Eluent: PE-EtOAc, 5:1. Colorless oil, yield 92%. $[\alpha]_D^{25}$ +66 (c 1.0, $CHCl_3$). UV λ_{max} (nm) (log ε) (MeOH): 201 (4.49). IR (neat), ν_{max} (cm^{-1}): 1751, 1506, 1370, 1211, 1114. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.07–7.14 (m, 2H, Ar−H), 7.05 (d, J = 1.7 Hz, 1H, Ar−H), 5.38 (dd, J = 8.9, 3.9 Hz, 1H, −C***H***($OCOCH_3$)−), 5.23 (br s, 1H, −NH−), 4.49–4.53 (m, 1H, −C***H***($COOCH_3$)−), 3.74 (2s, 3H, −$COOCH_3$), 3.31–3.42 (m, 2H, −SCH_2−), 3.14–3.18 (m, 1H, $ArCH_2$−), 3.03–3.07 (m, 1H, $ArCH_2$−), 2.28 (2s, 6H, $ArOCOCH_3$), 2.10 (s, 3H, −$OCOCH_3$), 1.44 (s, 9H, −$OC(CH_3)_3$). ^{13}C NMR (100 MHz, $CDCl_3$) δ 197.6, 170.7, 169.8, 169.7, 168.2, 168.2, 141.9, 141.1, 134.2, 127.5, 124.6, 123.4, 80.3, 78.1, 52.8, 37.3, 30.7, 29.7, 28.3, 20.7, 20.6. MS (ESI) *m*/*z*: 564.5 [M + Na^+]. HRMS calcd mass for $C_{24}H_{31}NO_{11}SNa$ [M + Na^+] 564.1510, found 564.1502.

*6.6.2. S-((R)-3-methoxy-3-oxo-2-(tert-butoxycarbonylamino)propyl) 2-acetoxy-3-(3,4-methylenedioxyphenyl)propanethioate (**5b**)*

Eluent: PE-EtOAc, 5:1. Yellow oil, yield 87%. $[\alpha]_D^{25}$ +135 (c 1.0, $CHCl_3$). UV λ_{max} (nm) (log ε) (MeOH): 207 (4.90), 235 (4.66), 287 (4.36). IR (neat), ν_{max} (cm^{-1}): 2927, 1748, 1697, 1490, 1443, 1367, 1216, 1162, 1037, 929, 756. ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.74 (d, J = 7.7 Hz, 1H, Ar−H), 6.69 (s, 1H, Ar−H), 6.65 (d, J = 7.7 Hz, 1H, Ar−H), 5.95 (s, 2H, −OCH_2O−), 5.34–5.37 (m, 1H, −C***H***($OCOCH_3$)−), 5.21 (br s, 1H, −NH−), 4.52–4.55 (m, 1H, −C***H***($COOCH_3$)−), 3.75 (s, 3H, −$COOCH_3$), 3.30–3.42 (m, 2H, −SCH_2−), 2.92–3.00 (m, 2H, $ArCH_2$−), 2.11 (s, 3H, −$OCOCH_3$), 1.45 (s, 9H, −$OC(CH_3)_3$). ^{13}C NMR (100 MHz, $CDCl_3$) δ 197.8, 170.8, 169.7, 169.6, 147.7, 146.7, 122.6, 109.7, 108.3, 101.0, 80.3, 78. 8, 52.8, 52.7, 37.9, 30.6, 28.3, 20.7. MS (ESI) *m*/*z*: 492.3 [M + Na^+]. HRMS calcd mass for $C_{21}H_{27}NO_9SNa$ [M + Na^+] 492.1299, found 492.1298.

*6.6.3. S-((R)-3-methoxy-3-oxo-2-(tert-butoxycarbonylamino)propyl) 2-acetoxy-3-(3,4-diacetoxyphenyl)propenethioate (**6a**)*

Eluent: PE-EtOAc, 3:1. White solid, mp 121–123 °C, yield 86%. $[\alpha]_D^{25}$ +32 (c 1.0, $CHCl_3$). UV λ_{max} (nm) (log ε) (MeOH): 203 (3.83), 248 (4.27), 300 (3.80). IR (neat), ν_{max} (cm^{-1}): 1772, 1714, 1503, 1369, 1261, 1160, 1113, 1014, 757. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.49 (s, 1H, Ar−H), 7.43 (d, J = 8.6 Hz, 1H, Ar−H), 7.22–7.24 (m, 2H, Ar−H and −CH═), 5.23 (br d, 1H, −NH−), 4.58 (br s, 1H, −C***H***($COOCH_3$)−), 3.77 (s, 3H, −$COOCH_3$), 3.49–3.52 (m, 2H, −SCH_2−), 2.34 (s, 3H, −$OCOCH_3$), 2.31 (s, 6H, 2 × $ArOCOCH_3$), 1.45 (s, 9H, −$OC(CH_3)_3$). ^{13}C NMR (100 MHz, $CDCl_3$) δ 186.6, 170.8, 167.9, 155.1, 143.2, 142.6, 142.3, 130.2, 128.5, 125.0, 124.1, 123.9, 80.3, 53.0, 52.8, 31.4, 30.2, 29.7, 28.3, 20.7. ESI-MS *m*/*z*: 562.3 [M + Na^+]. HRMS calcd mass for $C_{24}H_{29}NO_{11}SNa$ [M + Na^+] 562.1354, found 562.1360.

*6.6.4. S-((R)-3-methoxy-3-oxo-2-(tert-butoxycarbonylamino)propyl) 2-acetoxy-3-(3,4-methylenedioxyphenyl)propenethioate (**6b**)*

Eluent: PE-EtOAc, 5:1. Yellow oil, yield 89%. $[\alpha]_D^{25}$ +40 (c 0.5, $CHCl_3$). UV λ_{max} (nm) (log ε) (MeOH): 202 (4.26), 250 (3.88), 342 (4.15). IR (neat), ν_{max} (cm^{-1}): 1715, 1504, 1261, 1160, 1038. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.21 (s, 1H, ArCH═), 7.12 (d, J = 1.5 Hz, 1H, Ar−H), 7.06 (dd, J = 8.2, 1.5 Hz, 1H, Ar−H), 6.83 (d, J = 8.2 Hz, 1H, Ar−H), 6.01 (s, 2H, −OCH_2O−), 5.30 (d, J = 7.8 Hz, 1H, −NH−), 4.53–4.58 (m, 1H, −C***H***($COOCH_3$)-), 3.76 (s, 3H, −$COOCH_3$), 3.41–3.52 (m, 2H, −SCH_2−), 2.36 (s, 3H, −$OCOCH_3$), 1.44 (s, 9H, −$OC(CH_3)_3$). ^{13}C NMR (100 MHz, $CDCl_3$) δ 186.5, 170.9, 168.0, 155.1, 149.5, 148.2, 140.6, 126.8, 126.1, 125.7, 109.2, 108.8, 101.7, 80.3, 53.1, 52.8, 31.2, 20.8. ESI-MS *m*/*z*: 490.2 [M + Na^+]. HRMS calcd mass for $C_{21}H_{25}NO_9SNa$ [M + Na^+] 490.1142, found 490.1156.

*6.6.5. S-((R)-3-methoxy-3-oxo-2-(tert-butoxycarbonylamino)propyl) 3-(3,4-diacetoxyphenyl)propanethioate (**18a**)*

Eluent: PE-EtOAc, 5:1. Colorless oil, yield 82%. $[\alpha]_D^{25}$ +26 (c 1.0, $CHCl_3$). UV λ_{max} (nm) (log ε) (MeOH): 202 (4.46). IR (neat), ν_{max} (cm^{-1}): 1773, 1714, 1506, 1369, 1213, 1182. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.10 (d, J = 8.3 Hz, 1H, Ar−H), 7.05 (dd, J = 8.3, 2.0 Hz, 1H, Ar−H), 7.00 (d, J = 2.0 Hz, 1H, Ar−H), 5.24 (br d, 1H, −NH−), 4.51–4.55 (m, 1H, −C***H***($COOCH_3$)−), 3.73 (s, 3H, −$COOCH_3$), 3.30–3.39 (m, 2H, −SCH_2−), 2.85–2.98 (m, 4H, −CH_2CH_2Ar), 2.28 (s, 6H, 2 × $ArOCOCH_3$), 1.44 (s, 9H, −$OC(CH_3)_3$). ^{13}C NMR (100 MHz, $CDCl_3$) δ 197.1, 170.9, 168.4, 168.3, 155.1, 142.0, 140.6, 138.6, 126.5, 123.4, 123.3, 80.2, 77.3, 53.0, 52.7, 44.9, 31.2, 30.6, 28.3, 20.6. MS (ESI) *m*/*z*: 506.5 [M + Na^+]. HRMS calcd mass for $C_{22}H_{29}NO_9SNa$ [M + Na^+] 506.1455, found 506.1449.

*6.6.6. S-((R)-3-methoxy-3-oxo-2-(tert-butoxycarbonylamino)propyl) 3-(3,4-methylenedioxyphenyl)propanethioate (**18b**)*

Eluent: PE-EtOAc, 5:1. Colorless oil, yield 79%. $[\alpha]_D^{25}$ +54 (c 1.0, $CHCl_3$). UV λ_{max} (nm) (log ε) (MeOH): 204 (4.66), 233 (4.14), 287 (3.79). IR (neat), ν_{max} (cm^{-1}): 1712, 1491, 1442, 1366, 1245, 1164, 1038. ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.72 (d, J = 7.9 Hz, 1H, Ar−H), 6.66 (s, 1H, Ar−H), 6.62 (d, J = 7.9 Hz, 1H, Ar−H), 5.92 (s, 2H, −OCH_2O−), 5.22 (d, J = 7.8 Hz, 1H, −NH−), 4.49–4.54 (m, 1H, -C***H***($COOCH_3$)-), 3.74 (s, 3H, −$COOCH_3$), 3.27–3.40 (m, 2H, −SCH_2−), 2.80–2.90 (m, 4H, −CH_2CH_2Ar), 1.44 (s, 9H, −$OC(CH_3)_3$). ^{13}C NMR (100 MHz, $CDCl_3$) δ 197.4, 170.9, 155.1, 147.7, 146.1, 133.5, 121.2, 108.8, 108.3, 100.9, 80.2, 77.3, 53.0, 52.7, 45.7, 31.2, 31.1, 28.3, 28.3. MS (ESI) *m*/*z*:

434.2 [M + Na^+]. HRMS calcd mass for $C_{19}H_{25}NO_7SNa$ [M + Na^+] 434.1244, found 434.1244.

6.7. General procedure for the thioester conjugates **5c–d**, **6c–d** and **18c–d**

To a solution of thioester conjugates **5a–b**, **6a–b** or **18a–b** (0.2 mmol) in CH_2Cl_2 (8 mL) was added trifluoroacetic acid (46 mg, 0.4 mmol) at 0 °C. The mixture was stirred at 10 °C for 6 h. The solution was kept at 0 °C and neutralized with saturated saturated $NaHCO_3$ (aq.) to pH 8–9, then extracted with CH_2Cl_2 (3 × 6 mL). The combined organic layers were washed with water (10 mL), brine (10 mL) and dried over anhydrous Na_2SO_4. After the removal of the solvent under the reduced pressure, the residue was submitted to silica-gel column chromatography separation to afford the corresponding product **5c–d**, **6c–d** or **18c–d**.

6.7.1. S-((R)-3-methoxy-3-oxo-2-aminopropyl) 2-acetoxy-3-(3,4-diacetoxyphenyl)propanethioate (**5c**)

Eluent: PE-EtOAc, 2:1. Colorless oil, yield 85%. $[\alpha]_D^{25}$ +5.5 (c 0.5, $CHCl_3$). UV λ_{max} (nm) (log ε) (MeOH): 202 (4.44), 276 (3.12). IR (neat), ν_{max} (cm^{-1}): 2926, 1744, 1672, 1528, 1443, 1373, 1228, 763. ^1H NMR (400 MHz, $CDCl_3$) δ 7.03–7.11 (m, 3H, Ar–H), 6.87 (d, J = 7.0 Hz, 2H, $-NH_2$), 5.33–5.42 (m, 1H, $-CH(OCOCH_3)-$), 4.76–4.79 (m, 1H, $-CH(COOCH_3)$-), 3.74, 3.76 (2s, 3H, $-COOCH_3$), 3.08–3.24 (m, 2H, $-SCH_2-$), 2.84–2.97 (m, 2H, $ArCH_2-$), 2.26 (s, 6H, 2 × $ArOCOCH_3$), 2.13, 2.17 (2s, 3H, $-OCOCH_3$). ^{13}C NMR (100 MHz, $CDCl_3$) δ 196.6, 170.2, 169.8, 169.7, 169.3, 147.1, 134.8, 128.0, 122.3, 121.9, 118.6, 73.6, 53.0, 52.8, 37.0, 30.4, 21.0, 20.9, 20.8. MS (ESI) m/z: 459.4 [M + NH_4^+]. HRMS calcd mass for $C_{19}H_{23}NO_9SNa$ [M + Na^+] 464.1030, found 464.1032.

6.7.2. S-((R)-3-methoxy-3-oxo-2-aminopropyl) 2-acetoxy-3-(3,4-methylenedioxyphenyl)propanethioate (**5d**)

Eluent: PE-EtOAc, 2:1. Colorless oil, yield 89%. $[\alpha]_D^{25}$ −1.8 (c 0.5, acetone). UV λ_{max} (nm) (log ε) (MeOH): 203 (4.56), 257 (2.85), 285 (3.55). IR (neat), ν_{max} (cm^{-1}): 3339, 2926, 1741, 1675, 1490, 1443, 1245, 1038, 930. ^1H NMR (400 MHz, $CDCl_3$) δ 6.80 (br s, 2H, $-NH_2$), 6.61–6.74 (m, 3H, Ar–H), 5.93 (s, 2H, $-OCH_2O-$), 5.29–5.39 (m, 1H, $-CH(OCOCH_3)-$), 4.79–4.83 (m, 1H, $-CH(COOCH_3)-$), 3.76, 3.78 (2s, 3H, $-COOCH_3$), 2.88–3.12 (m, 4H, $ArCH_2$- and $-SCH_2-$), 2.13, 2.18 (2s, 3H, $-OCOCH_3$). ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.1, 169.3, 168.5, 147.7, 146.8, 129.0, 122.8, 110.0, 108.3, 101.0, 77.2, 74.1, 53.3, 52.9, 37.2, 21.0. MS (ESI) m/z: 370.0 [M + H^+]. HRMS calcd mass for $C_{16}H_{19}NO_7SNa$ [M + Na^+] 392.0774, found 392.0783.

6.7.3. S-((R)-3-methoxy-3-oxo-2-aminopropyl) 2-acetoxy-3-(3,4-diacetoxyphenyl)propenethioate (**6c**)

Eluent: PE-EtOAc, 1:1. Colorless oil, yield 92%. $[\alpha]_D^{25}$ +20.2 (c 1.0, $CHCl_3$). UV λ_{max} (nm) (log ε) (MeOH): 202 (4.27). IR (neat), ν_{max} (cm^{-1}): 2925, 1745, 1505, 1434, 1371, 1206, 1181, 1112, 1014, 901. ^1H NMR (400 MHz, $CDCl_3$) δ 7.45 (d, J = 1.8 Hz, 1H, Ar–H), 7.39 (dd, J = 8.7, 1.8 Hz, 1H, Ar–H), 7.21 (d, J = 8.7 Hz, 1H, Ar–H), 7.20 (s, 1H, =CHAr), 6.95 (d, J = 6.5 Hz, 2H, $-NH_2$), 4.92–4.97 (m, 1H, $-CH(COOCH_3)-$), 3.83 (s, 3H, $-COOCH_3$), 3.05–3.14 (m, 2H, $-SCH_2-$), 2.36 (s, 3H, $-OCOCH_3$), 2.30 (s, 6H, 2 × $ArOCOCH_3$). ^{13}C NMR (100 MHz, $CDCl_3$) δ 174.2, 170.8, 169.7, 168.3, 168.2, 141.8, 141.0, 134.6, 127.6, 124.8, 123.2, 78.2, 52.9, 52.8, 38.8, 34.6, 20.7. MS (ESI) m/z: 462.4 [M + Na^+]. HRMS calcd mass for $C_{19}H_{21}NO_9SNa$ [M + Na^+] 462.0974, found 462.0978.

6.7.4. S-((R)-3-methoxy-3-oxo-2-aminopropyl) 2-acetoxy-3-(3,4-methylenedioxyphenyl)propenethioate (**6d**)

Eluent: PE-EtOAc, 1:1. Colorless oil, yield 94%. $[\alpha]_D^{25}$ −0.8 (c 0.2, $CHCl_3$). UV λ_{max} (nm) (log ε) (MeOH): 203 (4.43), 288 (3.87), 320 (3.86). IR (neat), ν_{max} (cm^{-1}): 3346, 2925, 1744, 1641, 1504, 1448, 1247, 1037. ^1H NMR (400 MHz, $CDCl_3$) δ 7.17 (s, 1H, =CHAr), 7.09 (s, 1H, Ar–H), 7.03 (d, J = 8.3 Hz, 1H, Ar–H), 6.90 (d, J = 7.0 Hz, 2H, $-NH_2$), 6.82 (d, J = 8.3 Hz, 1H, Ar–H), 6.00 (s, 2H, $-OCH_2O-$), 4.92–4.94 (m, 1H, $-CH(COOCH_3)-$), 3.82 (s, 3H, $-COOCH_3$), 3.05–3.14 (m, 2H, $-SCH_2-$), 2.38 (s, 3H, $-OCOCH_3$). ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.4, 168.1, 162.2, 159.1, 148.1, 140.9, 129.8, 125.6, 124.4, 110.0, 108.9, 101.5, 53.7, 53.0, 31.9, 22.7. MS (ESI) m/z: 368.2 [M + H^+]. HRMS calcd mass for $C_{16}H_{17}NO_7SNa$ [M + Na^+] 390.0618, found 390.0613.

6.7.5. S-((R)-3-methoxy-3-oxo-2-aminopropyl) 3-(3,4-diacetoxyphenyl)propanethioate (**18c**)

Eluent: PE-EtOAc, 2:1. Colorless oil, yield 91%. $[\alpha]_D^{25}$ +15 (c 0.5, $CHCl_3$). UV λ_{max} (nm) (log ε) (MeOH): 200 (4.52), 202 (3. 65). IR (neat), ν_{max} (cm^{-1}): 1770, 1658, 1507, 1372, 1214. ^1H NMR (400 MHz, $CDCl_3$) δ 7.10 (s, 2H, Ar–H), 7.05 (s, 1H, Ar–H), 6.31 (d, J = 7.5 Hz, 2H, $-NH_2$), 4.85–4.87 (m, 1H, $-CH(COOCH_3)-$), 3.77 (s, 3H, $-COOCH_3$), 2.92–3.00 (m, 4H, $ArCH_2$- and $-SCH_2-$), 2.54–2.61 (m, 2H, $-CH_2COS-$), 2.25, 2.27 (2s, 6H, 2 × $ArOCOCH_3$). ^{13}C NMR (100 MHz, $CDCl_3$) δ 197.6, 171.5, 170.6, 168.4, 142.0, 140.4, 139.4, 126.6, 123.4, 123.3, 52.8, 52.1, 37.5, 30.8, 30.5, 20.7. MS (ESI) m/z: 406.3 [M + Na^+]. HRMS calcd mass for $C_{17}H_{21}NO_7SNa$ [M + Na^+] 406.1058, found 406.1056.

6.7.6. S-((R)-3-methoxy-3-oxo-2-aminopropyl) 3-(3,4-methylenedioxyphenyl)propanethioate (**18d**)

Eluent: PE-EtOAc, 5:1. Colorless oil, yield 88%. $[\alpha]_D^{25}$ +28 (c 0.5, $CHCl_3$). UV λ_{max} (nm) (log ε) (MeOH): 203 (4.21), 285 (3.25). IR (neat), ν_{max} (cm^{-1}): 1743, 1652, 1490, 1442, 1246, 1039. ^1H NMR (400 MHz, $CDCl_3$) δ 6.64–6.74 (m, 3H, Ar–H), 6.26 (br d, 2H, $-NH_2$), 5.92 (s, 2H, $-OCH_2O-$), 4.86–4.88 (m, 1H, $-CH(COOCH_3)-$), 3.78 (s, 3H, $-COOCH_3$), 2.88–2.97 (m, 4H, $-SCH_2-$ and $ArCH_2-$), 2.47–2.56 (m, 2H, $ArCH_2CH_2-$). ^{13}C NMR (100 MHz, $CDCl_3$) δ 171.7, 170.5, 147.7, 146.1, 134.2, 121.2, 108.9, 108.4, 100.9, 53.4, 52.8, 38.5, 31.3, 26.9. MS (ESI) m/z: 312.1 [M + H^+]. HRMS calcd mass for $C_{14}H_{17}NO_5SNa$ [M + Na^+] 334.0720, found 334.0722.

6.8. Cell culture and treatment

Human umbilical vein endothelial cells (HUVECs) were obtained from ATCC (Manassas, VA). Cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 μg/mL streptomycin at 37 °C in a humidified atmosphere comprised of 95% air and 5% CO_2. Passage 3–8 was used for experiments. Cells were exposed to different Danshensu-cysteine analog conjugates (5, 50, 100 μmol/L) or NAC (5 mmol/L) for 4 h, and then subjected to 200 μmol/L H_2O_2 for 12 h.

6.9. Determination of cell viability

Cell viability analysis was performed based on the capacity of mitochondrial enzymes to transform 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan. HUVECs were seeded in 96-well plate (150 μL DMEM with 10% FBS per well) and incubated overnight. After growing to sub-confluence, cells were pretreated with various concentrations of Danshensu-cysteine analog conjugates (5, 50, 100 μmol/L) in DMEM without FBS for 4 h. After that, H_2O_2 with a final 200 μmol/L concentration was added to the culture medium for 12 h. Next, 100 μL of MTT (5 mg/mL) was added to each well and cells were incubated at 37 °C for 4 h. Then, the culture medium with MTT was abandoned and the colored formazan was dissolved in 100 μL of dimethyl sulfoxide (DMSO). The absorption values were measured at 570 nm using

a microplate reader (Tecan infinite 200). The viability of HUVECs in each well was presented as percentage of control cells.

6.10. Preparation of cell lysates

The cells were grown to confluence in 24-well plates. After drug treatment the culture supernatant was collected for analysis of LDH. The cells were scraped from the plates into ice-cold RIPA lysis buffer (Beyotime, China) and protein concentration was determined by bicinchoninic acid protein assay kit (Biocolor Bioscience & technology company, Shanghai, China). Aliquots were stored at −80 °C until detection for MDA and GSH level.

6.11. LDH, MDA, GSH measurement

The LDH leakage, level of MDA and activity of GSH were all determined by using commercially available kits. All the measurements were performed according to the manufacturer's protocols.

6.12. Flow cytometric evaluation of cell apoptosis and mitochondrial membrane potential

HUVECs growing in 60 mm dishes were harvested, washed and double-stained by using an Annexin V-FITC apoptosis detection kit. Samples were quantitatively analyzed by a flow cytometer (BD, FACSCalibur).

Mitochondrial membrane potential of HUVECs was assessed by using JC-1 Assay Kit. After the drug treatment, total cells were collected into 2 mL tubes and incubated with JC-1 for 20 min at 37 °C. Finally, cells were washed, harvested, re-suspended with JC-1 staining buffer and analyzed on a flow cytometer as before.

6.13. Fluorescent staining of HUVECs with Hoechst 33258

Hoechst 33258 is a kind of blue fluorescent dye which is sensitive to DNA conformation and chromatin state in cells. Consequently, Hoechst 33258 can be used to detect the degree of nuclear damage. Briefly, cells were washed with PBS, fixed with 3.7% paraformaldehyde for 10 min at room temperature, washed twice with PBS and then stained with Hoechst 33258 for 20 min at room temperature. After three washes with PBS, cells were observed under a confocal microscope (LSM 510, ZEISS).

6.14. Western blot analysis

HUVECs were washed with ice-cold PBS and lysed in RIPA lysis buffer (Beyotime, China) for 15 min on ice, and cell lysate was centrifuged at 12,000 g for 10 min at 4 °C. The supernatant was collected and protein content of extracted samples was measured using bicinchoninic acid protein assay kit (Biocolor Bioscience & technology company, Shanghai, China). Equal amount of proteins (30 μg) was subjected to 8–12% SDS-polyacrylamide gel electrophoresis and transferred to PVDF membranes (Millipore Corporation). The membranes were blocked with 5% nonfat dried milk in 0.1% Tween 20 in Tris-buffered saline (TBST) for 1 h, and incubated with primary antibodies (Bax, Blc-2, caspase-3, p53, PARP, caspase-9 and β-actin) overnight at 4 °C. Antibodies were detected by means of HRP-conjugated secondary antibody for 1 h at room temperature. Immunoreactive bands were visualized using enhanced chemiluminescence reagents (Beyotime, China) and densitometric analysis was performed with the use of Alpha Image (Alpha Innotech, USA).

6.15. Statistic analysis

Data were presented as means ± S.D. and analyzed by SPSS software. Two-tailed Student's *t*-test was carried out to determine statistical significance. Differences were considered significant at $P < 0.05$.

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