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Asymmetric synthesis and biological evaluation of Danshensu derivatives as anti-myocardial ischemia drug candidates

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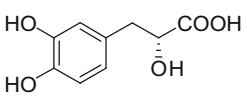
ABSTRACT

The synthesis and bioactivities of Danshensu derivatives (*R*)-methyl 2-acetoxy-3-(3,4-diacetoxyphenyl)propanoate (**1a**), (*R*)-methyl 2-acetoxy-3-(3,4-methylenedioxyphenyl)propanoate (**1b**) and their racemates **7** and **10** were reported in this paper. These derivatives were designed to improve their chemical stability and liposolubility by protecting Danshensu's phenolic hydroxyl groups with acetyl or methylene which could be readily hydrolyzed to release bioactive Danshensu. The asymmetric synthesis of **1a** and **1b** were achieved by catalytic hydrogenation of (*Z*)-methyl 2-acetoxy-3-(3,4-diacetoxyphenyl)-2-propenoate (**6a**) and (*Z*)-methyl 2-acetoxy-3-(3,4-methylenedioxyphenyl)-2-propenoate (**6b**) in excellent enantiomeric excesses (92% *ee* and 98% *ee*, respectively) and good yields (>89%). An unexpected intermediate product, (*Z*)-2-acetoxy-3-(3,4-dihydroxyphenyl)acrylic acid (**4c**) was obtained with high chemoselectivity in 86% yield by keeping the reaction temperature at 60 °C and its structure was identified by X-ray single crystal diffraction analysis. **1a**, **1b** and their racemates **7**, **10** as well as **4c** exhibited potent protective activities against hypoxia-induced cellular damage. The in vitro test showed that all these compounds could increase cell viability, and inhibit lipid hyperoxidation. Furthermore, **1a** and **4c** could inhibit apoptosis by regulating the expression of apoptosis-related molecule in gene and protein levels, up-regulating the expression of bcl-2 and down-regulating bax and caspase-3. The in vivo test indicated that **4c** exhibited anti-myocardial ischemic effects featured by reducing infarction size and increasing the level of the intracellular enzymes detectable in serum. Therefore, these Danshensu derivatives may be good drug candidates for anti-myocardial ischemia therapy and merit further investigation.

1. Introduction

Danshensu is an active component of Danshen, the dried root of *salvia miltiorrhiza* mostly responsible for many biological activities, such as dilating coronary arteries, inhibiting platelet aggregation, myocardial cell apoptosis and anti-inflammatory property.[1–3] These activities are at least partially related to its anti-oxidative activity.[4] Accordingly, Danshensu itself and its scaffold are privileged and of great interest as synthetic targets and building blocks for cardiovascular active drug candidates.



Danshensu

Isolation and purification of Danshensu from natural resources are difficult and time consuming due to the chemical unstability of phenolic hydroxyl groups, low content in Danshen and a variety of structural analogs as impurities. Several synthetic strategies have been explored to produce (±)-Danshensu,[5,6] but asymmetric synthesis of Danshensu is extremely rare. Findrik developed a mathematical model for the enzymatic kinetics of the synthesis of (*R*)-(+)-3,4-dihydroxyphenyllactic acid, which was catalyzed by *D*-lactate dehydrogenase from *Lactobacillus leishmannii*.[7] The preparation of Danshensu using a chemical method based on catalytic asymmetric hydrogenation has never been reported. Moreover, being a hydrophilic molecule and thus poorly soluble in lipidic matrices, Danshensu uneasily enters into cells by crossing the cell membranes. Therefore, it is highly desirable to search for new Danshensu derivatives with potent pharmacological activity, good stability and liposolubility.

Danshensu has the structure of phenyllactic acid bearing two phenolic hydroxyl groups on its phenyl ring which are considered as the radical scavenging moieties. The presence of different substituents in the phenol backbone structure may modulate their antioxidant property. In general, monophenol is a less efficient

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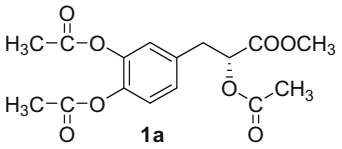
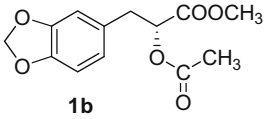
antioxidant than polyphenol. The introduction of electron donating groups such as hydroxyl in the *ortho* or *para* position increases the antioxidant activity of phenolic acid.[8,9] In addition, the presence of a carbonyl group, such as aromatic acid, ester, or lactone enhanced antioxidant activity. Steric hindrance of the phenolic hydroxyls by a neighboring inert group, such as methoxyl group, enhanced its antioxidant activity.[10] According to such structure-activity relationship, Danshensu derivatives (**1a**, **1b**) were designed with the phenolic hydroxyls esterized by acetylation or protected by methylene. Such structures could enhance the stability and liposolubility, and is beneficial to the chiral catalysts used in the asymmetric reduction step. These structural modification of Danshensu were expected to exhibit similar pharmacological activity with Danshensu due to the ester and ether linkages are easily hydrolyzed and readily release bioactive Danshensu.[11–15]



1a



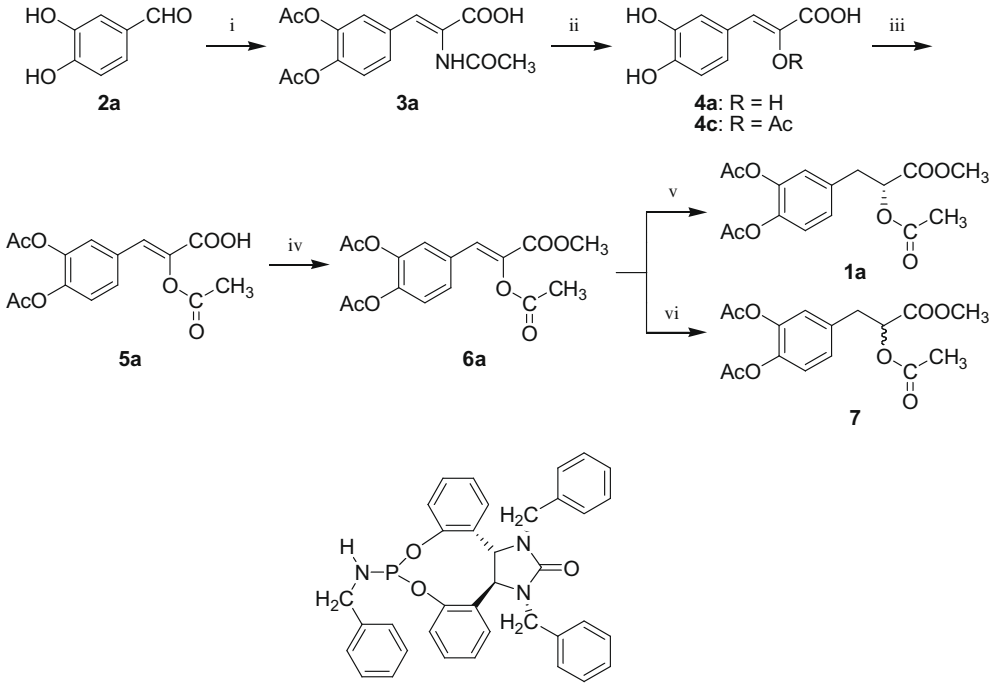
1b

Moreover, it is also attractive to study the pharmacological difference between the enantiomers and racemates. Herein, we report the efficient asymmetric synthesis of **1a**, **1b** and an unexpected intermediate product **4c** and their preliminary pharmacological assay results.

2. Chemistry

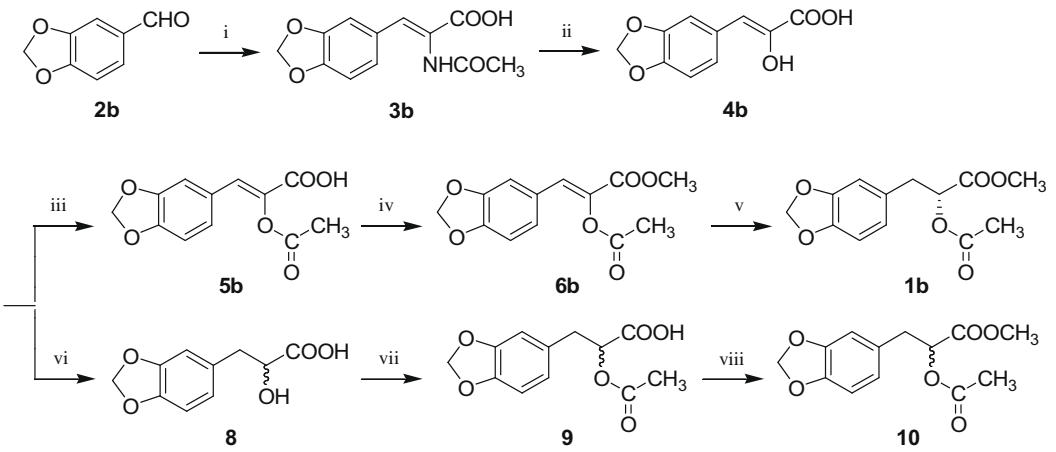
The designed compounds were synthesized efficiently according to the procedures outlined in Schemes 1 and 2. 3,4-Dihydroxybenzaldehyde (**2a**) and its analog piperonal (**2b**) were chosen as the starting material, and **3a** and **3b** were easily accessible by the condensation of **2a** and **2b** with *N*-acetylglycine followed by hydrolysis in hydrochloric acid to give the key intermediates **4a** and **4b**, respectively.[16,17]

To prepare the enantiomers of Danshensu derivatives, two approaches were studied. In the first strategy, compound **4b** was subjected to hydrogenation in the presence of catalytic amount of Pd–C and cinchonidine as the chiral ligand, but the enantiomeric excess of the product was very low (16% *ee*).[18] Recently the excellent performance of monophosphites, monophosphonites, and monophosphoramidites as ligands provide tremendous interest and potential in asymmetric hydrogenation.[19] Thus, to improve the enantiomeric excess of product, in the second approach, a new generation of the monodentate phosphoramidites, compound **11** developed by Ding and co-workers[19] was employed as the chiral ligand and $[Rh(cod)_2]BF_4$ as the catalyst precursor in asymmetric hydrogenation. First, compounds **4a** and **4b** were protected at the OH-groups by esterization with acetic anhydride in the presence of sodium acetate to provide the corresponding products **5a** and **5b** in 80% and 77% yields, respectively. Followed by the reaction with diazomethane in ice-cooled dichloromethane, the thoroughly protected compounds **6a** and **6b** were obtained in 81% and 85% yields, respectively. Using **11** as chiral ligand, under the modified conditions of the literature procedures,[19] excellent enantioselectivities (92–98% *ee*) and good yields (>89%) of target compounds **1a** and **1b** were realized. Racemic compounds **7** and **10** were also synthesized. First, compound **8** was obtained by two methods of reduction of **4b** with KBH_4 and catalytic hydrogenation. The condensation of compound **8** with acetic anhydride in the presence of sodium acetate gave compound **9** in 95% yield. Compound **10** was attained by reacting **9** with diazomethane in ice-cooled dichloromethane in 79% yield. Using Pd/C as catalyst, racemic compound **7** was prepared by catalytic hydrogenation of compound **6a** in 79%



Chiral ligand (**11**)

Scheme 1. The synthetic route of Danshensu derivatives **1a**, **4c** and **7**. Reaction conditions: (i) *N*-acetylglycine, acetic anhydride, NaOAc, 100 °C, 2 h, pour into cold water; (ii) **4a**: 9% HCl, 100 °C, 6 h; **4c**: 9% HCl, 60 °C, 3 h; (iii) acetic anhydride, NaOAc, rt, 5.5 h; (iv) CH_2N_2, dichloromethane, 0 °C; (v) $[Rh(cod)_2]BF_4$, chiral ligand **11**, H_2, 60 atm, CH_2Cl_2, rt, 24 h; (vi) Pd–C, EtOAc, 10 atm, 24 h.



Scheme 2. The synthetic route of Danshensu derivatives **1b** and **10**. Reaction conditions: (i) *N*-acetylglycine, acetic anhydride, NaOAc, 100 °C, 3 h; add water, 120 °C, 0.5 h; (ii) 9% HCl, 100 °C, 4 h; (iii) acetic anhydride, NaOAc, rt 7 h; (iv) CH_2N_2, CH_2Cl_2, 0 °C; (v) $[Rh(cod)_2]BF_4$, chiral ligand **11**, H_2, 60 atm, CH_2Cl_2, rt, 24 h; (vi) Pd–C, H_2, 6 atm, MeOH, 24 h; or KBH_4/MeOH, rt, 7 h; (vii) acetic anhydride, HOAc, rt, 24 h; (viii) CH_2N_2, CH_2Cl_2, 0 °C.

yield. Interestingly, the hydrolyzation of **3a** was found to lead to different products at different temperatures. **4a** was got at 100 °C for 6 h and at a lower temperature of 60 °C for 3 h compound **4c** was obtained in 86% yield. However, we were unable to identify the position of acetyl group simply on the basis of its ^1H NMR, ^{13}C NMR, and MS spectra data. The single crystals of **4c** were obtained by recrystallization from water and analyzed by X-ray single crystal diffraction (Fig. 1). Unexpectedly, 2-acetamido in **3a** was converted to 2-acetoxy and 3′,4′-diacetoxy groups hydrolyzed to 3′,4′-dihydroxyls to form **4c**. This result can be rationalized as follows: 2-acetamido and 3′,4′-diacetoxy groups was easily hydrolyzed to hydroxyls at low temperature, however acetyl recombined to 2-dihydroxyl to get 2-acetoxy, and such bonding was so tight that could be hydrolyzed only at high temperature and lasting time.

3. Pharmacological assay results

3.1. Protective effects of compounds 1a, 1b, 7, 10, 4c on hypoxia-induced neonatal rat ventricular myocytes (NRVMs)

The protective effects of compounds **1a**, **1b**, **7**, **10** and **4c** were investigated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide(MTT) assay and LDH leakage assay. Cells were incubated with indicated concentrations of compounds **1a**, **1b**, **7**, **10** and **4c** for 12 h before exposure to hypoxia, then cells were subjected to hypoxia for 5 h. Cell viability was assessed by MTT reduction assay. It showed that compounds **1a**, **1b**, **7**, **10** and **4c** at 1 μmol/L significantly increased cell viability compared with hypoxia group (Fig. 2). The marker of cell damage LDH leakage was assessed after hypoxia and it was found that compounds **1a**, **1b**,

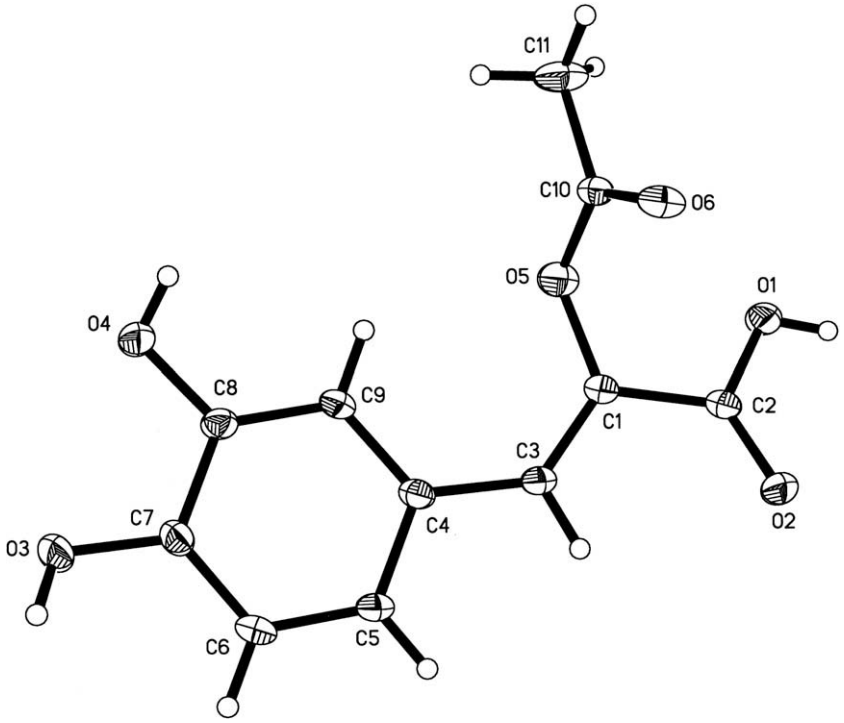


Figure 1. The single crystal structure of compound **4c**.

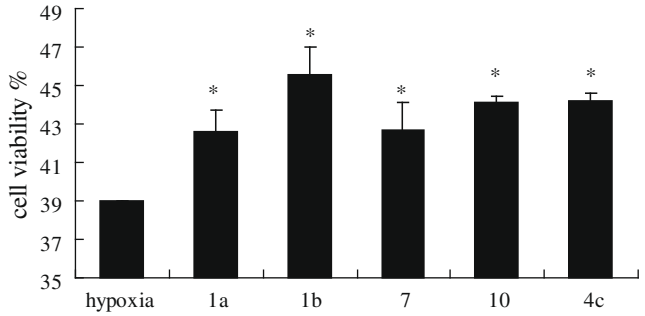


Figure 2. Effects of compounds **1a**, **1b**, **7**, **10** and **4c** at 1 μmol/L on cell viability in hypoxia-induced neonatal rat ventricular myocytes. Compounds **1a**, **1b**, **7**, **10** and **4c** could significantly increase cell viability. Values are expressed as means ± SE from six individual examples. $^{*}P < 0.05$ versus hypoxia.

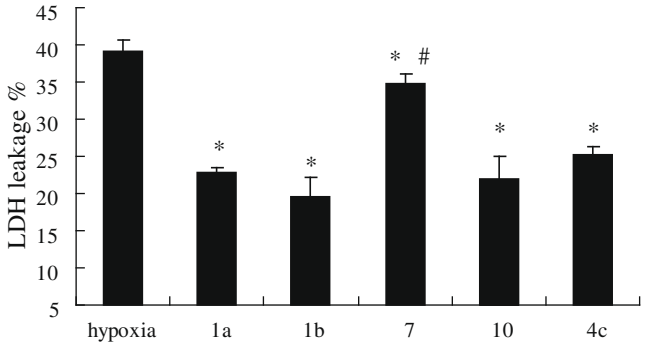


Figure 3. Effects of compounds **1a**, **1b**, **7**, **10** and **4c** at 1 μmol/L on LDH leakage in hypoxia-induced neonatal rat ventricular myocytes. Values are expressed as means ± SE from six individual examples. $^{*}P < 0.05$ versus hypoxia, $^{\#}P < 0.05$ between compounds **1a** and **7**.

7, **10** and **4c** at 1 μmol/L could decrease LDH leakage (Fig. 3). At the same concentration of 1 μmol/L, compounds **1a** and **1b** possessed higher activity than racemic compounds **7** and **10** in decreasing cellular damage. Therefore, the antioxidant property of compounds **1a**, **1b** and **4c** was further evaluated.

3.2. Antioxidant activity of compounds 1a, 1b and 4c in hypoxia-induced neonatal rat ventricular myocytes

To determine the antioxidant activity of compounds **1a**, **1b** and **4c**, malondialdehyde (MDA), a marker of oxidant-mediated lipid peroxidation in cells was quantified after hypoxia. As illustrated in Figure 4, when compounds **1a**, **1b** and **4c** were served at 1 μmol/L, MDA content was decreased compared with the hypoxia group ($P < 0.05$), indicating that compounds **1a**, **1b** and **4c** could inhibit the lipid peroxidation of cell membrane.

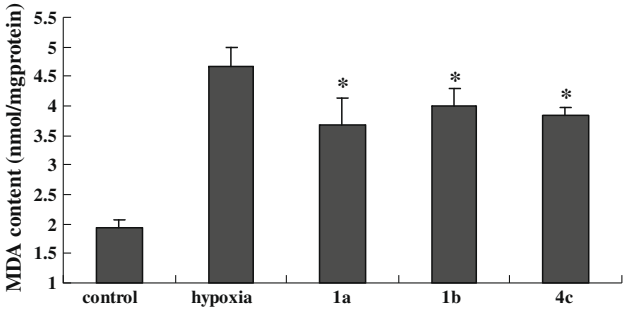


Figure 4. Effects of compounds **1a**, **1b**, **4c** at 1 μmol/L on MDA content in hypoxia-induced neonatal rat ventricular myocytes. Values are expressed as means ± SE from six individual examples. $^{*}P < 0.05$ versus hypoxia.

3.3. Anti-apoptotic activity of compounds 1a and 4c in hypoxia induced neonatal rat ventricular myocytes

Apoptosis is a programmed cell death that is characterized by specific structural changes that include cell shrinkage, nuclear condensation and DNA fragmentation. To assess the anti-apoptotic activity of **1a** and **4c**, Hoechst staining was performed to observe the morphological changes in hypoxia-induced neonatal rat ventricular myocytes. Administration of compounds **1a** and **4c** resulted in less nuclei-shrunk and nuclear condensation compared with the hypoxia group (Fig. 5).

At the molecular level, apoptosis is activated by the aspartate-specific cysteine protease cascade, including caspase-3 and caspase-12. Caspase-3 is considered to be the most important executioner caspases and is activated by any of the initiator caspases.[20] Besides caspases, members of the bcl-2 family are also critical for the regulation of apoptosis. The relative concentration of these proteins in the outer mitochondrial membrane is thought to determine the survival or death of a cell following an apoptotic stimulus.[21] To further elucidate the mechanism of anti-apoptotic activity of **1a** and **4c**, their effects on modulating the expression of various apoptosis-related biomarkers, including caspase-3, bax and bcl-2 were investigated. RT-PCR assay showed that the level of bax and capase-3 mRNA in hypoxia-induced neonatal rat ventricular myocytes declined significantly after treatment by compounds **1a** and **4c** relative to hypoxia group ($P < 0.05$), whereas the level of bcl-2 mRNA markedly increased (Fig. 6). Western blot showed consistent results with RT-PCR in protein level (Fig. 7).

3.4. In vivo protective effects of 4c on acute myocardial ischemia in adult rat

Considering the readily synthesis and potent pharmacological activity in vitro of **4c**, its cardio-protective effects in rats of acute

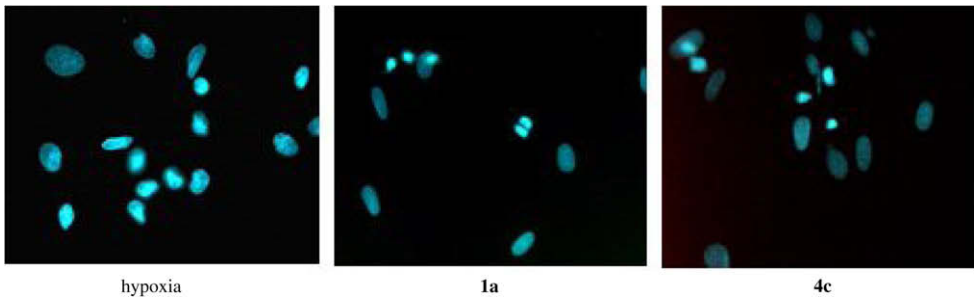


Figure 5. Effects of compounds **1a**, **4c** at 1 μmol/l on apoptosis in hypoxia-induced neonatal rat ventricular myocytes. Nuclear staining was achieved using Heochst dye.

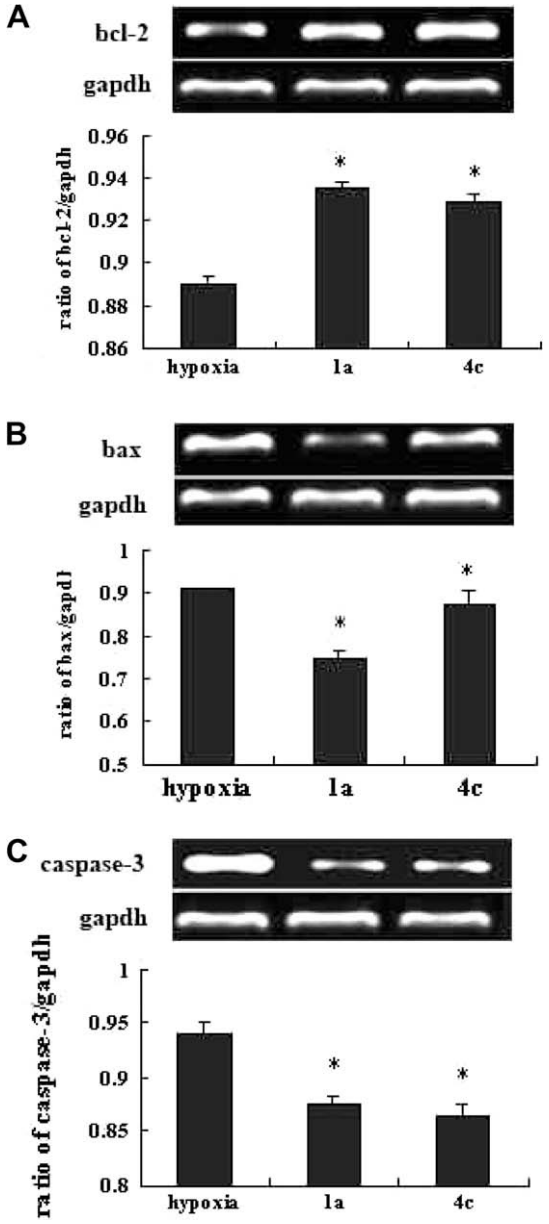


Figure 6. Effects of compounds **1a**, **4c** at 1 μmol/l on the expression of bcl-2, bax, caspase-3 gene in hypoxia-induced neonatal rat ventricular myocytes. Quantitative analysis of the mRNA of bcl-2 (A), bax (B) and caspase-3 (C). The value at each group represents the normalized mean ± SE from six individual examples. $^{*}P < 0.05$ versus hypoxia.

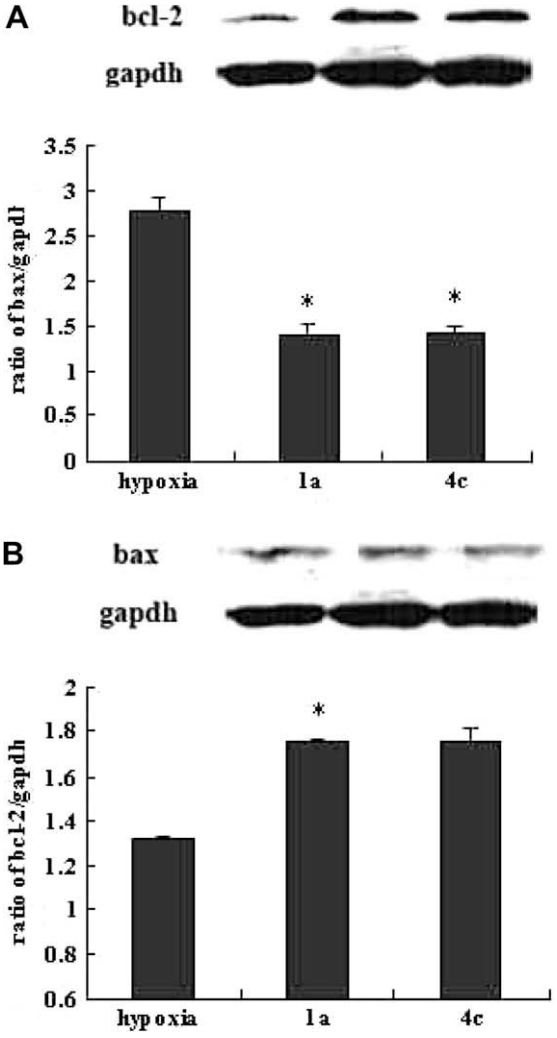


Figure 7. Effects of compounds **1a**, **4c** at 1 μmol/l on the expression of bcl-2, bax, protein in hypoxia-induced neonatal rat ventricular myocytes. Quantitative analysis of the protein of bcl-2 (A) and bax (B). The value at each group represents the normalized mean ± SE from six individual examples. $^{*}P < 0.05$ versus hypoxia.

myocardial infarction were further evaluated. Rats were treated with **4c** at different concentrations (15, 30 and 60 mg/kg). Two days after myocardial infarction surgery, the hearts were analyzed by TTC staining to quantify infarct size. As shown in Figure 8, the infarct areas in compound **4c**-treated rats were smaller than in the model rats, and only the difference between the rats treated with **4c** at 60 mg/kg and model rats was of statistic significance. The level of clinical markers of cardiac infarction in serum was also measured. As expected, the levels of serum lactate dehydrogenase (LDH), creatine kinase (CK) in model rats were dramatically higher compared with those in the sham-operated rats, which indicated that myocardial infarction was successfully established. Compared with model rats, the levels of serum LDH, CK and MDA in rats treated with **4c** were significantly reduced, meanwhile the activity of SOD in serum was increased in **4c** treated rats (Fig. 9), which

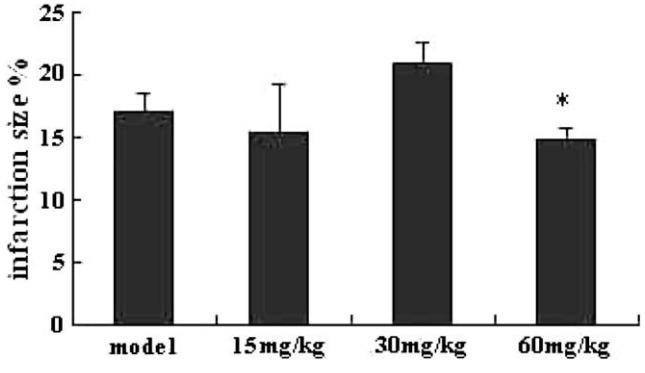


Figure 8. Effects of compound **4c** at different concentrations on infarction size in rat after myocardial infarction. Values are expressed as means ± SE from six individual examples. $^{*}P < 0.05$ versus model.

was consistent with the results of infarction area, indicating that **4c** treatment apparently promoted cell integrity and reduced myocardial damage.

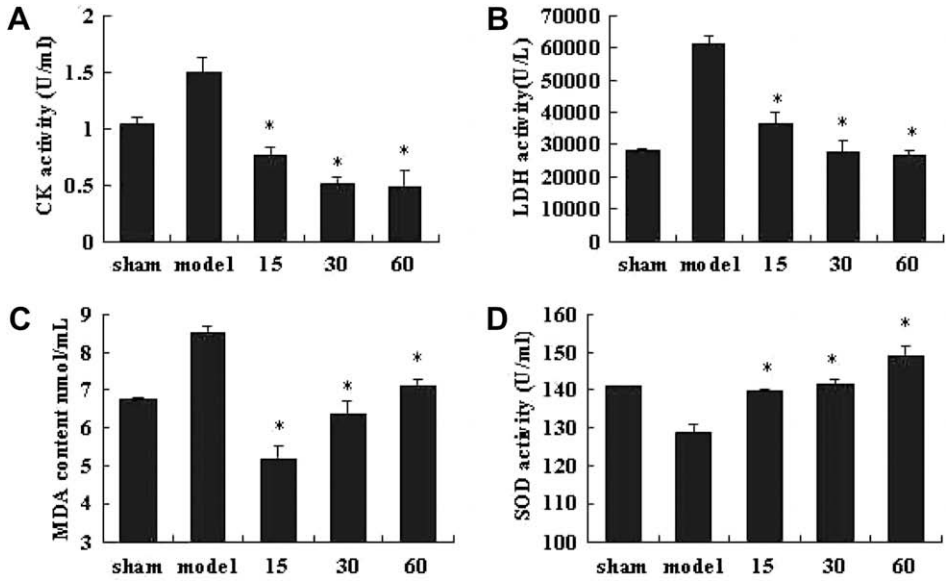


Figure 9. Effects of compound **4c** at concentrations of 15, 30, 60 mg/kg on the levels of CK (A), LDH (B), MDA (C) and SOD (D) in serum. Values are expressed as means ± SE from six individual examples. $^{*}P < 0.05$ versus model.

4. Discussion

Danshensu, the only component of Danshen that has been studied in human subjects, exhibited well-known pharmacological activity. However, it is hard to obtain either from natural Danshen due to the unstability of phenolic hydroxyl or from chemical synthesis due to its chiral structure. In the present study, several novel Danshensu derivatives were designed and synthesized asymmetrically. The hydroxyl groups in these Danshensu derivatives were protected by acetyl or methylene, which not only increased their stability and liposolubility but also was beneficial to asymmetric synthesis and pharmacological assay. These structural modifications of Danshensu were expected to exhibit similar pharmacological activity with Danshensu due to both the ester and ether linkages are easily hydrolyzed and readily release bioactive Danshensu. Using a chemical approach based on asymmetric hydrogenation, (*R*)-Danshensu derivatives **1a** and **1b** as well as their racemates **7** and **10** were successfully obtained in excellent *ee* and high yields.

Compounds **1a**, **1b**, **7**, **10** and **4c** significantly increased cell viability and reduced LDH leakage, which signified that these compounds possessed potent protective effects against hypoxia-induced cellular damage. At the same concentration of 1 μmol/L, the LDH leakage of (*R*)-**1a** treated cells was less than that of cells treated by **7**, indicating that the bioactivity may be related to the configuration of the chiral carbon and *R*-configuration was more favorable. However, the difference of LDH leakage between (*R*)-**1b** and **10** treated cells was not significant, suggesting that different substitutions of phenolic hydroxyl groups influence on the metabolism in vivo and further impact the pharmacological activity.

Compounds **1a**, **1b** and **4c** possessed potent antioxidant activity featured by decreased MDA content, which may be related to the hydrolysis to expose phenolic hydroxyl groups due to the cleavage of the labile ester bond. However, there may be another way for these compounds to act as antioxidant agents, for example increasing the activity of intracellular antioxidant enzyme. Therefore, the mechanism of antioxidative activity of **1a**, **1b** and **4c** merits further investigations.

Using Hoechst staining, compounds **1a** and **4c** showed potent anti-apoptotic activity. From our results, the mechanism by which **1a** and **4c** inhibit cell apoptosis was possibly through suppressing apoptotic signaling. Systemic levels of anti-apoptotic protein bcl-2 were significantly higher in cells treated with compounds **1a** and **4c**, pro-apoptotic protein bax and apoptosis executive protein caspase-3 were lower in **1a** and **4c** treated group compared with hypoxia group. In addition, the anti-apoptosis activity of compounds **1a** and **4c** may be partially related to their antioxidant property.

The results in vivo showed that compound **4c** could protect cardiac myocytes from myocardial infarction injury by reducing infarct area and decreasing the level of the intracellular enzymes detectable in serum. Considering readily synthesis and purification as well as potent pharmacological activity, compound **4c** was promising for further therapeutic applications.

5. Conclusion

Overall, we successfully synthesized new derivatives of Danshensu, (*R*)-**1a**, (*R*)-**1b**, (±)-**7**, (±)-**10** and **4c**, which are more stable and liposoluble than Danshensu. Their structures were confirmed through NMR, MS and X-ray single crystal diffraction analysis. Pharmacological evaluation showed that these Danshensu derivatives possessed potent cardio-protective activities that occur through blocking oxidative stress and apoptotic pathways. Further structural modification, biological screening and the mechanism study are in progress as these promising pharmacological results demonstrate that these Danshensu derivatives merit attention as potential anti-myocardial ischemia drugs.

6. Experimental

Starting materials and reagents were obtained from commercial suppliers and were used without purification. Melting points were determined in open capillary tubes and are uncorrected. Nuclear magnetic resonance spectra were recorded on a Brucker-DPX 400 MHz spectrometer, Mass spectral data was collected on a HP5973 N analytical mass spectrometer. HRMS data were determined on an IonSpec 4.7 Tesla FTMS instrument. X-ray single crystal diffraction was carried out on a Bruker Smart CCD diffractometer.

6.1. (*Z*)-2-Acetamido-3-(3,4-diacetylphenyl)acrylic acid (3a)

3,4-Dihydroxybenzaldehyde (**2a**) (10.8 g, 0.078 mol), *N*-acetylglycine (9.2 g, 0.0789 mol), sodium acetate (6.4 g, 0.078 mol) and acetic anhydride (30 mL, 32.4 g, 0.319 mol) were mixed together and stirred at 140 °C for 2 h. After cooling, the dark-coloured viscous solution was poured into cold water (400 mL). The sticky yellow gum which separated hardened on keeping overnight. It was collected and crystallized from EtOAc to give **3a** as a yellow solid (12.1 g, 48%): mp 183–185 °C (lit.[16]: 183.4 °C). ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.08 (s, 3H, 2-NHCOCH_3), 2.28 (s, 6H, 3′-CH_3COO, 4′-CH_3COO), 7.06 (s, 1H, 3-H), 7.14 (m, 3H, 2′-H, 5′-H, 6′-H), 8.01 (s, 1H, 2-N*H*COCH$_3$).

6.2. (*Z*)-2-Acetamido-3-(3,4-methylenedioxyphenyl)acrylic acid (3b)

3,4-Methylenedioxyphenyl aldehyde (**2b**) (5.1 g, 0.034 mol), *N*-acetylglycine (5.20 g, 0.044 mol), sodium acetate (4 g, 0.049 mol) and acetic anhydride (20 mL, 21.6 g, 0.213 mol) were mixed together and stirred at 100 °C for 3 h. After completion of the reaction, the mixture was allowed to cool to rt. Then water (100 mL) was added, and stirred at 120 °C for 0.5 h. After cooling, the mixture was filtered and the filtrate was extracted with EtOAc. The organic extract was evaporated to give **3b** as a pale yellow solid (4.68 g, 55%): mp 215–216 °C (lit.[17]: 216–216.5 °C).

6.3. (*Z*)-3-(3,4-Dihydroxyphenyl)-2-hydroxyacrylic acid (4a)

A mixture of **3a** (1.0 g, 0.016 mol) in 9% HCl (40 mL) was refluxed at 100 °C for 6 h. Then the resulting mixture was allowed to cool to rt and filtered. The filtrate was extracted with EtOAc. The organic extract was evaporated. The crude product was dried under vacuum and washed with water to get **4a** as a slightly yellow solid (0.53 g, 87%): mp 179–181 °C (lit.[17]: 181 °C). ^{1}H NMR (400 MHz, acetone-d_6) δ 4.5 (br, 3H, OH), 6.44 (s, 1H, 2′-H), 6.81 (d, 1H, J = 8.2 Hz, 5′-H), 7.12 (d, 1H, J = 8.2 Hz, 6′-H), 7.50 (s, 1H, 3-H), 7.61 (br, 1H, COOH).

6.4. (*Z*)-3-(3,4-Methylenedioxyphenyl)-2-hydroxyacrylic acid (4b)

A mixture of **3b** (3 g, 0.012 mol) in 9% HCl (50 mL) was refluxed at 100 °C for 4 h. After that, the resulting mixture was allowed to cool to rt and extracted with EtOAc (3 × 30 mL). The organic extract was evaporated and the product was dried under vacuum and washed with water to get **4b** as a slightly yellow solid (2 g, 80%): mp 207 °C (lit.[17]: 206 °C). ^{1}H NMR (400 MHz, acetone-d_6) δ 5.93 (s, 2H, OCH_2O), 6.42 (s, 1H, 2′-H), 6.78 (d, 1H, J = 8.22 Hz, 6′-H), 7.11 (d, 1H, J = 8.41 Hz, 5′-H), 7.48 (s, 1H, 3-H).

6.5. 3-(3,4-Methylenedioxyphenyl)-2-hydroxypropanoic acid (8)

A mixture of **4b** (1.0 g, 4.8 mmol), Pd–C (0.21 g) and anhydrous MeOH (10 mL) was hydrogenated at 6 atm for 24 h. The mixture was filtered and concentrated, the residue was purified by silica gel chromatography, providing **8** as white solid (0.44 g, 43%): mp 96 °C (lit.[17]: 95–97 °C). ^{1}H NMR (400 MHz, acetone-d_6) δ 2.77–3.01 (m, 2H, 3-H), 4.28–4.31 (m, 1H, 2-H), 5.90(s, 2H, OCH_2O), 6.69 (s, 2H, 5′-H, 6′-H), 6.78 (s, 1H, 2′-H).

A mixture of **4b** (2 g, 9.6 mmol) and 25% methanol (40 mL) was adjusted pH to 8 by sodium hydroxide then was added KBH_4 (1.5 g, 27.8 mmol). The mixture was stirred at room temperature for 7 h and adjusted pH to 2 by 1 *N* HCl and then extracted with EtOAc (3 × 30 mL). The organic extract was concentrated and purified by silica gel chromatography providing **8** as white solid (1.41 g, 70%).

6.6. (*Z*)-2-Acetoxy-3-(3,4-diacetoxyphenyl)acrylic acid (5a)

A mixture of **4a** (0.9 g, 4.59 mmol) and sodium acetate (0.5 g, 6.1 mmol) in acetic anhydride (6 mL, 6.48 g, 0.064 mol) was stirred at rt for 5.5 h. After that, the solution was added water (15 mL), and was extracted with EtOAc (3 × 30 mL). The combined organic layers were dried over anhydrous $MgSO_4$. Concentration of the dried extracts yielded a residue which was purified by crystallization from acetic acid to give **5a** as yellow solid (1.2 g, 80%): mp 184–186 °C (lit.[22]: 189–192 °C). ^{1}H NMR (400 MHz, acetone-d_6) δ 2.28–2.29 (m, 9H, 2-$OCOCH_3$, 3′-$OCOCH_3$, 4′-$OCOCH_3$), 7.31 (d, 1H, J = 8.3 Hz, 5′-H), 7.34 (s, 1H, 3-H), 7.63 (d, 1H, J = 8.5 Hz, 6′-H), 7.64 (s, 1H, 2′-H).

6.7. (*Z*)-2-Acetoxy-3-(3,4-methylenedioxyphenyl)acrylic acid (5b)

A mixture of **4b** (1.0 g, 4.8 mmol) and sodium acetate (0.6 g, 7.3 mmol) in acetic anhydride (8 mL, 8.64 g) was stirred at rt for 5.5 h. The formed solid was filtered off, washed with water, air-dried, and crystallized from EtOAc to give **5b** as yellow solid (0.93 g, 77%): mp 164–166 °C; IR (KBr, cm^{-1}) 2960, 2936, 2853, 2522, 1756, 1691, 1639, 1500, 1444, 1429, 1265, 1245, 1199, 1116, 1039; ^{1}H NMR (400 MHz, acetone-d_6) δ 2.32 (s, 3H, 2-$OCOCH_3$), 6.08 (s, 2H, 3′,4′-OCH_2O), 6.92 (d, 1H, J = 7.83 Hz, 3-H), 7.24–7.28 (m, 3H, 2′-H, 6′-H, 5′-H). MS (EI) *m/z* (%): 250 (M^+, 6.44), 208 (39.85), 194 (9.29), 162 (100.00), 150 (6.67), 134 (39.25), 104(15.41), 76(27.58). HRMS calcd mass for $C_{12}H_{10}O_6$ 250.0477, found 250.0481.

6.8. 2-Acetoxy-3-(3,4-methylenedioxyphenyl)propanoic acid (9)

To a solution of **8** (0.4 g, 2.4 mmol) in acetic anhydride (2 mL, 2.16 mg, 0.021 mmol) was added acetic acid (1 mL) and the mixture was stirred at rt for 24 h. After that, the solution was added water (5 mL), and was extracted with EtOAc (3 × 10 mL). The combined organic layers were dried over anhydrous $MgSO_4$. Concentration of the dried extracts yielded a residue which was purified by crystallization from acetic acid to give **9** as white solid (0.45 g, 95%): mp 151–153 °C; IR (KBr, cm^{-1}) 2960, 2853, 2567, 1749, 1723, 1710, 1505, 1498, 1450, 1259, 1226, 1070, 1041; ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.09 (s, 3H, 2-$OCOCH_3$), 3.00–3.15 (m, 2H, 3-H), 5.15–5.18 (m, 1H, 2-H), 5.93 (s, 2H, 3′,4′-OCH_2O), 6.69 (d, 1H, J = 8.24 Hz, 6′-H), 6.73 (s, 1H, 2′-H), 6.74 (d, 1H, J = 7.74 Hz, 5′-H), 7.86 (br, 1H, COOH). MS (EI) *m/z* (%): 252 (M^+, 0.32), 192 (100.00), 175 (6.00), 135 (99.68), 105(6.57), 77(20.23). HRMS calcd mass for $C_{12}H_{12}O_6$ 252.0634, found 252.0644.

6.9. General procedure for the synthesis of 6a, 6b and 10

To an ice-cooled solution of **5a** or **5b** or **9** (0.02 mol) in dry dichloromethane (20 mL) was added a solution of diazomethane in ether dropwise with stirring. Drop adding was stopped until the solution was clear, and the solution was stirred overnight. After that, the mixture was concentrated and the residue was purified by silica gel chromatography to give **6a**, **6b**, **10**.

6.9.1. (*Z*)-Methyl 2-acetoxy-3-(3,4-diacetylphenyl)acrylate (6a)

White solid, yield 81%, mp 95–96 °C; IR (KBr, cm^{-1}) 3504, 2960, 2936, 2853, 1760, 1722, 1654, 1503, 1380, 1305, 1245, 1212, 1122,

1092; ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.30–2.32 (m, 9H, 2-$OCOCH_3$, 3′-$OCOCH_3$, 4′-$OCOCH_3$), 3.84 (s, 3H, $COOCH_3$), 7.22 (d, 1H, J = 8.22 Hz, 5′-H), 7.25(s, 1H, 3-H), 7.43 (d, 1H, J = 8.61 Hz, 6′-H), 7.48 (s, 1H, 2′-H). MS (EI) m/z (%): 336 (M^+, 0.60), 294 (25.11), 252 (77.88), 210 (96.24), 150 (100.00), 122 (11.07). HRMS calcd mass for $C_{16}H_{16}O_8$ 336.0845, found 336.0849.

6.9.2. (*Z*)-Methyl 2-acetoxy-3-(3,4-methylenedioxyphenyl)-acrylate (6b)

White solid, yield 85%, mp 84–85 °C; IR (KBr, cm^{-1}) 3061, 2954, 2914, 1773, 1708, 1654, 1615, 1508, 1489, 1338, 1282, 1244, 1195, 1099; ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.34 (s, 3H, 2-$OCOCH_3$), 3.83 (s, 3H, $COOCH_3$), 6.01 (s, 2H, 3′,4′-OCH_2O), 6.82 (d, 1H, J =7.82 Hz, 5′-H), 7.06 (d, 1H, J = 8.22 Hz, 6′-H), 7.16 (s, 1H, 3-H), 7.23 (s, 1H, 2′-H). MS (EI) m/z (%): 264 (M^+, 4.04), 222 (50.29), 162 (100.00), 134 (33.75), 134 (39.25), 104(6.30), 76(16.06). HRMS calcd mass for $C_{11}H_9O_5$ 221.0450, found 221.0442.

6.9.3. Methyl 2-acetoxy-3-(3,4-methylenedioxyphenyl)propanoate (10)

White solid, yield 79%, mp 63–65 °C; IR (KBr, cm^{-1}) 2957, 2920, 1760, 1736, 1491, 1448, 1376, 1297, 1253, 1239, 1197, 1038; ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.10(s, 3H, 2-$OCOCH_3$), 2.97–3.10(m, 2H, 3-H), 3.73(s, 3H, $COOCH_3$), 5.14–5.17 (m, 1H, 2-H), 5.94 (s, 2H, 3′,4′-OCH_2O), 6.66 (d, 1H, J = 7.72 Hz, 6′-H), 6.71 (s, 1H, 2′-H), 6.82 (d, 1H, J = 8.21 Hz, 5′-H).

6.10. General procedure for the synthesis of 1a and 1b

$[Rh(cod)_2]BF_4$ (2.4 mg, 0.006 mmol), ligand **11**[19] (7.7 mg, 0.0132 mmol) were dissolved in CH_2Cl_2 (1 mL) under nitrogen and the solution was stirred at room temperature for 10 min, the substrate (200 mg, 0.6 mmol) in CH_2Cl_2 (1.5 mL) was added to the above catalyst solution, the mixture was then transferred to a stainless steel autoclave under nitrogen atmosphere, and then sealed. After purging with hydrogen for three times, final H_2 pressure was adjusted to 60 atm. After stirring at room temperature for 24 h, H_2 was released, the mixture was filtered and the filtrate was concentrated. The residue was purified by silica gel chromatography to give compounds **1a** and **1b**.

6.10.1. (*R*)-Methyl 2-acetoxy-3-(3,4-diacetoxyphenyl)propanoate (1a)

White solid, yield 89%, *ee* 92%, mp 81–82 °C, $[\alpha]_D^{20} = +3.6$ (c = 1.0 in $CHCl_3$); IR (KBr, cm^{-1}) 2963, 1771, 1755, 1735, 1504, 1377, 1205, 1178; ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.09 (s, 3H, 2-$OCOCH_3$), 2.28 (s, 6H, 3′-$OCOCH_3$, 4′-$OCOCH_3$), 3.05–3.18 (m, 2H, 3-H), 3.72 (s, 3H, $COOCH_3$), 5.17–5.20 (m, 1H, 2-H), 7.07 (s,1H, 2′-H), 7.12 (s, 2H, 5′-H, 6′-H). MS (EI) m/z (%): 338 (M^+, 0.66), 278 (3.95), 254 (7.28), 236 (17.92), 194 (100.00), 163 (8.56), 123(22.04). HRMS calcd mass for $C_{16}H_{18}O_8$ 338.1002, found 338.1013.

The enantiomeric excess was determined by HPLC on Chiralpak AS-H column, hexane:isopropanol = 90:10; flow rate = 1.0 mL/min; UV detection at λ = 254 nm; t = 30.6 min (minor), 34.5 min (major), respectively.

6.10.2. (*R*)-Methyl 2-acetoxy-3-(3,4-methylenedioxyphenyl)-propanoate (1b)

White solid, yield 89%, *ee* 98%, mp 70–71 °C, $[\alpha]_D^{20} = +6.2$ (c = 1.0 in $CHCl_3$); IR (KBr, cm^{-1}) 3014, 2965, 2920, 1753, 1734, 1490, 1239, 1219, 1033; ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.10 (s, 3H, 2-$OCOCH_3$), 3.01–3.08 (m, 2H, 3-H), 3.73 (s, 3H, $COOCH_3$), 5.14–5.17 (m, 1H, 2-H), 5.95 (s, 2H, 3′,4′-OCH_2O), 6.66 (d, 1H, J = 7.82 Hz, 6′-H), 6.71 (s, 1H, 2′-H), 6.74 (d, 1H, J = 7.82 Hz, 5′-H). MS (EI) m/z (%): 266 (M^+, 12.19), 206 (100.00), 193 (4.15), 175 (41.89), 135 (97.92), 105(6.21), 77(18.38). HRMS calcd mass for $C_{13}H_{14}O_6$ 226.0790, found 226.0806.

The enantiomeric excess was determined by HPLC on Chiralpak AD-H column, hexane:isopropanol = 95:5; flow rate = 0.7 mL/min; UV detection at λ = 214 nm; t = 17.5 min (major), 22.3 min (minor), respectively.

6.11. Methyl 2-acetoxy-3-(3,4-diacetoxyphenyl)propanoate (7)

A mixture of **6a** (0.2 g, 0.59 mmol), Pd–C (30 mg) and EtOAc (4 mL) was hydrogenated at 10 atm for 24 h. The mixture was filtered and concentrated, the residue was purified by silica gel chromatography, providing **7** as white solid (160 mg, 79%): mp 64 °C; IR (KBr, cm^{-1}) 2963, 1771, 1754, 1736, 1503, 1377, 1205, 1178; ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.09 (s, 3H, 2-$OCOCH_3$), 2.28 (s, 6H, 3′-$OCOCH_3$, 4′-$OCOCH_3$), 3.05–3.18 (m, 2H, 3-H), 3.72 (s, 3H, $COOCH_3$), 5.17–5.20 (m, 1H, 2-H), 7.06 (s, 1H, 2′-H), 7.12 (s, 2H, 5′-H, 6′-H).

6.12. (*Z*)-2-Acetoxy-3-(3,4-dihydroxyphenyl)acrylic acid (4c)

A mixture of **3a** (2 g, 0.012 mol) in 9% HCl (50 mL) was heated at 60 °C for 3 h. The formed solid was filtered off, washed with water, air-dried, and crystallized from water to give **4c** as a colorless slice (1.27 g, 86%): mp 229–230 °C; IR (KBr, cm^{-1}) 3258, 3037, 1686, 1636, 1617, 1521, 1245, 1225, 1200, 1172, 1118; ^{1}H NMR (400 MHz, CD_3OD) δ 2.13 (s, 3H, 2-$OCOCH_3$), 6.77 (d, 1H, J = 8.21 Hz, 5′-H), 6.96 (d, 1H, J = 8.41 Hz, 2′-H), 7.16 (d, 1H, J = 1.95 Hz, 6′-H), 7.38 (s, 1H, 3-H). ^{13}C NMR (100 MHz, CD_3OD) δ 22.58, 116.28, 117.55, 123.50, 124.89, 126.60, 137.09, 146.35, 148.81, 168.68, 173.46. MS (ESI) m/z: 238 (M^+).

Crystal with dimensions of 0.421 mm × 0.347 mm × 0.250 mm for compound **4c** was selected and mounted on a Bruker Smart CCD diffractometer with graphite monochromatized Mo $K\alpha$ radiation (λ = 0.071073 nm). Diffraction data were collected using $\omega - 2\theta$ scans at room temperature (293 K). A perspective view of the structure is depicted in Figure 1.

Empirical formula: $C_{11}H_{10}O_6$. Formula weight: 238.19. Crystal system: monoclinic. Space group: $P2(1)/c$. Unit cell dimensions: a = 1.05986 (13) nm, b = 0.93423 (11) nm, c = 1.19601 (15) nm. V = 1.0751 (2) nm^3. Theta range for data collection is from 2.12° to 26.50°, Z = 4, D_c = 1.472 g/cm^3, $F(000)$ = 496. Refinement method: full-matrix least-squares on F^2. Goodness-of-fit on F^2: 1.075. Final R indices [$I > 2\sigma(I)$]: 0.0610, 0.1776. R indices (all data): 0.0700, 0.1858. Largest difference in peak and hole: 0.695 and −524 e/nm^3.

6.13. Cell culture

Neonatal rat ventricular myocytes (NRVMs) were isolated from 2- or 3-day old Spragur-Dawley rats and cultured as previously described.[23] Briefly, the hearts were removed and minced in PBS buffer. These tissue fragments were digested by trypsin dissociation. The dissociated cells were centrifuged for 5 min at 12,000 r/min. The pellets were re-suspended with DMEM supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin. After incubation for 2 h, the non-adherent cardiac myocytes were plated at a density of 1×10^6 cells/ml and seeded in plastic plates with 100 μM 5-bromo-deoxyuridine (Sigma) and incubated in a 5% CO_2 humidified incubator at 37 °C. After a period of 48 h, cells were exposed to different compounds for 12 h, and then subjected to hypoxia for 5 h.

6.14. Assessment of myocardial cells viability and damage

Cell viability was assessed by the measurement of the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to produce a dark blue formazan product that can be mea-

sured using a microplate reader at 570 nm. The viability of normal cell is presumed as 100%. Cell damage in cardiac myocytes was quantitatively assessed by the measurement of intracellular lactate dehydrogenase (LDH), it is presumed that in normal cell group there is no LDH leakage.

6.15. RNA isolation and RT-PCR

Total RNA was isolated from cardiac myocytes by using Trizol (Invitrogen, Carlbad, CA) as previously described. Reverse transcription(RT) was conducted by using PrimeScript™ 1st Strand cDNA Synthesis Kit according to the manufacture's procedures. Primers used for amplification were synthesized as follows: bcl-2: forward 5′-CGGGAGAACAGGGTATGA-3′; reverse 5′-CAGGCTG GAAGGA GAAGAT-3′. Bax: forward 5′-GCAGGGAGGATGGCTGGG GAGA-3′; reverse 5′-TCCAGACAAGCAGCCGCTCACG-3′. Caspase-3: forward 5′-CTGGACTGCGGTATTGAG-3′; reverse 5′-GGGTGCGGTAG AGTAAGC-3. GAPDH: forward 5′-TTCAACGGCACAGTCAAGG-3′; reverse 5′-CGGCATGTCAGATCCACAA-3. The PCR products were analyzed by electrophoresis in 1.5% agarose gels. The intensity of each band was photographed and quantified by using the Fluor Chem SP system (USA) as a ratio of a target gene over GAPDH.

6.16. Western blot

At the end of hypoxia, cardiac myocytes were scraped off in cell lysis buffer (Beyotime Biotechnology, Haimen, China) and centrifugated at 10,000 r/min for 10 min. The supernatant was collected, and protein concentrations were quantified using the enhanced BCA Protein Assay Kit (Beyotime Biotechnology, Haimen, China). Thirty micrograms of total proteins were separated on SDS–PAGE gels as previously described. Separated proteins were transferred onto 0.45-μm polyvinylidene difluoride (PVDF) membrane (Millipore Corporation). PVDF membrane was blocked in Tris-buffered saline (TBS) containing 5% skim milk for 2 h at room temperature and probed with rabbit anti-rat bcl-2 polyclonal antibody (R&D, USA) diluted in TBS-T for 1 h at room temperature. Anti-rabbit IgG diluted in TBS-T was used as secondary antibody. Protein band intensities were quantified by using a Western blotting detection system (Alpha Innotech, USA).

6.17. Model of myocardial infarction

The method was used as described previously.[24] Briefly, male Sprague-Dawley rats weighing 230–250 g, provided by the experimental animal center of fudan university were intubated and artificially ventilated with a rodent ventilator (DHX-150, China) under anesthesia with 7% choral hydrate (60 mg/kg ip). The normal electrocardiogram (II) was recorded after electrodes were placed subcutaneously and connected to an electrocardiograph. Then the chest was opened by left thoracotomy in the 3rd and 4th intercostal space and the pericardium was removed. The left anterior descending coronary artery was ligated with a 5–0 suture 1–2 mm below the left atrial appendage. Sham operated rats underwent an identical surgical procedure except that the left coronary was not ligation. Rats were randomly divided into five groups: MI group, sham-operated group, MI and drug-treated groups (15, 30, 60 mg/kg). The drug was injected intraperitoneally 7 days before surgery and 2 days after surgery once daily. Successful MI model was confirmed by pallor of the anterior wall of the left ventricle and ST-segment elevation.

6.18. Measurement of infarct size

The heart was excised immediately 2 days after the coronary artery was ligated and the entire ventricular tissue was sliced into five sections through the transverse axis from the apex to the atrioventricular groove and incubated in 1% triphenyltetrazolium chloride (TTC) at 37 °C for 15 min. Viable myocardium is stained in red by TTC, whereas infarction tissue remains unstained. Slices were imaged and the area of infracted myocardium was defined using an image analysis software (Scion Image, CA, USA). The size of the infarction area was estimated by the volume as a percentage of the left ventricle.

6.19. Measurements of lactate dehydrogenase (LDH), creatine kinase (CK), superoxide dismutase (SOD), malondialdehyde (MDA)

The levels of LDH, CK, SOD, MDA in serum were measured using diagnostic kit (NJBI, China) according to the manufacture's protocol.

6.20. Statistic analysis

Data were presented as means ± SE and analyzed by SPSS software. Pictures were processed with Photoshop software. Differences at $P < 0.05$ were considered statistically significant.

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