Synthesis and Biological Evaluation of Danshensu and Tetramethylpyrazine Conjugates as Cardioprotective Agents

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Myocardial ischemia is a primary cause of sudden death worldwide. Numerous active ingredients of traditional Chinese medicines including danshensu (DSS) and tetramethylpyrazine (TMP) have been widely used for the treatment of myocardial ischemia. To enhance their therapeutic efficacy and improve their druggability, in this work, we designed new DSS and TMP conjugates. Their water solubility and protective effects were studied in vitro and in experimental animal models. The new compounds demonstrated higher activities than the positive control agents acetylated danshensu and tetramethylpyrazine conjugate (ADTM) and salvianolic acid B (SAB) in preventing cells from oxidative insult. Among the new compounds, compound 14 was much more potent in preventing cells from oxidative injury, at least 10- and 20-fold as potent as ADTM and SAB, respectively. The protective effects of compound 14 may be attributed to its anti-radical activity and anti-apoptotic activity. These results suggest that compound 14 is a promising candidate for the treatment of myocardial ischemia.

Key words  danshensu; tetramethylpyrazine; synthesis; water solubility; myocardial ischemia

Myocardial ischemia is a primary cause of sudden death worldwide. Myocardial ischemia results from a decreased blood flow to the heart, preventing the heart from receiving adequate supply of oxygen. The reduced blood flow is usually the result of a partial or complete blockade of the arteries. Traditional treatments focus on restoring blood supply, such as by pass-graft surgery, dilation of coronary artery and coronary thrombolysis, and etc.5)

Although restoration of blood flow to the ischemic heart tissues is important, it is not adequate because myocardial ischemic damage is progressively developed following reperfusion due to an overproduction of a large number of biospecies.2,3) Compelling evidences demonstrated that free radicals generated during ischemia and reperfusion play pivotal roles in myocardial apoptosis. Free radical scavengers can effectively inhibit myocardial apoptosis.4,6) Previously, studies have demonstrated that agents that combine anti-thrombotic and anti-oxidative activities showed promising therapeutic efficacy in the treatment of myocardial ischemia.7)

In China, many traditional herbs have been used to prevent and treat myocardial ischemia. Among them are Salvia miltiorrhiza (Danshen) and Ligation Wallachia Franchat (Chuanxiong). As the major active ingredients, danshensu (3-(3,4-dihydroxy-phenyl) lactic acid) (DSS; Fig. 1), isolated from danshen, and tetramethylpyrazine (TMP; Fig. 1), isolated from Chuanxiong, have a variety of biological activities. They lyse blood clot, dilate coronary arteries, inhibit platelet aggregation, scavenge free radicals, improve microcirculation and have anti-inflammatory properties.8–14) Both of them are widely used in clinic for treatment of heart disease. Although widely used, their therapeutic benefits are severely limited due to their weak activities.

To increase the cardioprotective effects of DSS and TMP, we have previously synthesized a series of DSS and TMP conjugates. We found that the compounds containing DSS and TMP linked via an ester bound displayed higher activities than DSS and TMP.15) Among them, acetylated danshensu and tetramethylpyrazine conjugate (ADTM) (Fig. 1) was found to reduce infarct size in a rat model of myocardial ischemia.15–17) However, ADTM was quickly hydrolyzed by carboxylesterase and its half-life was too short in vivo.18) In addition, ADTM has poor water solubility. These shortcomings reduce the enthusiasms for further development of ADTM as a clinical candidate.

To improve the stability of DSS and TMP conjugates in blood, we found that introduction of allyl groups to the ester bond between DSS and TMP significantly extended the half-life. Although some compounds displayed higher cardioprotective effects than ADTM in cultured cells, their protective effects were not as good as that of ADTM in a rat model of acute myocardial ischemia (data not shown).19) Recently, we have reported several novel conjugates incorporating DSS, TMP and hydrogen sulfide donors.20) Some of these compounds displayed higher activities in preventing cells from oxidative insult, unfortunately, all of them have poor water solubility.

Fig. 1. Structures of DSS, TMP, ADTM and New DSS–TMP Conjugates

5: R₁=COCH₃, R₂=COCH₃
6: R₁=H, R₂=COCH₃
7: R₁=H, R₂=H
10: R₁=COCH₂NH₂, R₂=COCH₃
11: R₁=COCH₂NH₂, R₂=H
12: R₁=COCH₃, R₂=H
14: R₁=COCH₃, R₂=COCH₂NH₂
Based on our previous findings, in this work, to develop new DSS and TMP conjugates with higher therapeutic efficacy and improved water solubility, new DSS and TMP conjugates were designed and synthesized. Their biological activity, water solubility and the mechanisms of action were investigated.

**Results and Discussion**

**Drug Design and Chemical Synthesis**

Structures of the new DSS and TMP conjugates were shown in Fig. 1. The free phenolic hydroxyl groups of DSS were the functional groups for scavenging free radicals. However, the free phenolic hydroxyl groups make DSS unstable in the air. To explore a balance between the stability and free radical scavenging activities, compound 7 bearing two free phenolic hydroxyl groups and compounds 5, 6, 10–12 and 14 with two protected phenolic hydroxyl groups were designed. We previously also reported that modifications at the alcoholic hydroxyl group affect the efficacy; therefore, in the new DSS and TMP conjugates, the alcoholic hydroxyl groups were substituted with different moieties. In order to increase water solubility of the new compounds, the glycine moiety(ies) was (were) introduced (compounds 10, 11, 14). In addition, based on previous findings, to enhance stability in blood, allyl groups were introduced to the ester bond formed by DSS and TMP.

The target compounds 5–7, and 10–11 were synthesized following the procedures illustrated in Chart 1. The methyl group of TMP was oxidized by KMnO₄ to give a carboxylic acid, which was then esterified by treatment with ethanol to produce compound 1. Compound 1 was treated with allyl bromide to afford compound 2 in 34% yield. The hydroxyl groups of DSS were protected by acetyl groups by treatment with acetic anhydride, and the resulting intermediate was reacted with oxalyl chloride to afford compound 4. Without further purification, compound 4 was coupled with compound 2 in the presence of n-butyllithium to afford compound 5. Compounds 6 and 7 were obtained by a controlled hydrolysis of compound 5 in aqueous Na₂CO₃ solution. The phenolic hydroxyl groups of compounds 6 and 7 were esterified by treatment with N-tert-butoxycarbonyl (Boc)-glycine in the presence of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDCI) and 1-hydroxybenzotriazole (HOBT) to afford compounds 8 and 9. The N-Boc protective groups of compounds 8 and 9 were cleaved in trifluoroacetic acid (TFA) to afford compounds 10 and 11, respectively. The procedures for the synthesis of compounds 12 and 14 were illustrated in Chart 2. The phenolic hydroxyl groups of compound 6 was esterified by treatment with acetic anhydride to give compound 12, which was reacted with N-Boc-glycine to afford compound 13. Removal of the N-Boc protective group in TFA

**Chart 1. Synthesis of Compounds 5–7 and 10–11**

**Chart 2. Synthesis of Compounds 12 and 14**
afforded the target compound 14.

**Water Solubility Assay** Water solubility is an important factor for pharmaceuticals, especially for those administered via intravenous injection. In this work, we tested the water solubility of new DSS and TMP conjugates by analysis of the mass of compounds in saturated solution using HPLC. The results were shown in Table 1. Compared with ADTM, introduction of two allyl groups into the ester bond between DSS and TMP significantly reduced the water solubility. Since two allyl groups predominantly determine the polarity of the whole molecule, the free phenolic hydroxyl groups failed to increase the water solubility (compound 6: 0.015 g/L versus compound 5: 0.079 g/L). In contrast, introduction of the glycine moieties either to the phenolic hydroxyl groups (compound 10) or the alcoholic hydroxyl group (compound 14) significantly improved the water solubility. The results indicated the water solubility of DSS and TMP conjugates can be improved by introduction of a free amino group.

**Biological Evaluation**

**Protective Effects against tert-Butyl Hydroperoxide (t-BHP)-Induced Cell Damage in H9c2 Cells**

Since oxidative stress caused by free radicals after myocardial ischemia plays pivotal roles in myocardial damage, we first investigated the protective effects of the new compounds in H9c2 cells subjected to t-BHP damage using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. As shown in Fig. 2, all of the new compounds displayed higher protective effects than the positive control 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. As shown in Fig. 2, all of the new compounds showed a good activity in inhibiting the expression of apoptosis-related proteins. As shown in Fig. 4, the level of Bcl-2 in cells treated with t-BHP was reduced compared with the control group, while the level of Bax was increased significantly. In contrast, pretreatment with compound 14 notably restored the level of Bcl-2, and decreased the expression of Bax. Caspase-3 is a member of the cysteine-aspartic acid protease (caspase) family. Sequential activation of cleaved caspase-3 plays a central role in the execution-phase of cell apoptosis. Similarly, t-BHP enhanced the activation of cleaved caspase-3, while the activation was reversed by treatment with compound 14 (Fig. 5). Cytochrome c released from mitochondria into the cytoplasm is a key step of apoptosis process. As shown in Fig. 6, compound 14 inhibited the release of cytochrome c notably. These results implied that compound 14 might partially prevent oxidative stress induced myocardial apoptosis through the intrinsic apoptotic pathway.

**Effect on Acute Myocardial Ischemia in Rats**

The therapeutic effect of compound 14 was further evaluated in a rat acute ischemia model. The increased water

![Fig. 2. Protective Effects on H9c2 Cells Subjected to t-BHP](image)

H9c2 cells were pre-incubated with compounds at indicated concentrations for 1 h, and then exposed to 150 µM t-BHP. Cell viability was measured at 24 h post-t-BHP challenge by using the MTT assay. Data were presented as the mean±standard deviation (S.D.) of three independent experiments. ***p<0.01 versus control group, ***p<0.01 versus t-BHP control group.
The solubility of compound 14 made it feasible to give rats via intravenous (i.v.) injection. Salvianolate injection (the main active ingredient was salvianolic acid B) was used as positive control. As shown in Fig. 7, compound 14 (30 mg/kg) significantly reduced the infarct size in a dose dependent manner at doses ranging from 3 to 30 mg/kg. By contrast, compound 14 showed a similar protective effect to salvianolate injection at the same dose of 10 mg/kg. Compound 14’s great protection in vivo and its greatly improved solubility indicated that it may be a promising drug candidate for the treatment of cardiovascular incidents.

Experimental
Chemistry Unless otherwise noted, all chemicals and solvents were purchased as reagent grade from commercial suppliers and used without further purification. 1H-NMR (300 MHz) and 13C-NMR (75 MHz) were recorded on a Bruker Avance 300 spectrometer. Chemical shifts are reported as δ value in parts per million (ppm) relative to tetramethylsilan (TMS) as internal standard. Coupling constants were reported in units of Hertz (Hz). Electrospray ionization (ESI)-MS were obtained in the positive ion detection mode on a Finnigan LCQ Advantage MAX mass spectrometer (Applied Biosystems, 4000 Q TRAP). High resolution (HR)-MS were obtained on a Waters Vevo G2 Q-Tof mass spectrometer. Elemental analysis was performed at Harmonia Biotechnology Co., Ltd. (Tianjin, China).

Ethyl 3,5,6-Trimethylpyrazine-2-carboxylate (1) To 200 mL of water was added TMP (15g, 110 mmol) and K2MnO4 (21g, 133 mmol). The mixture was stirred at 50°C for 10h, followed by cooling to room temperature. The mixture was filtered and the residue was washed with H2O (5 mL). The filtrate was extracted with ethyl acetate (3×100 mL) and...
the remaining water layer wasacidified to pH 2–3 with dilute hydrochloric acid. The water layer was extracted with ethyl acetate (5×).

Solvent was removed in vacuo. To the residue, diethylamine (1.8 g, 147 mmol) was added and stirred until the reaction was completed as monitored by TLC. The pH of the mixture was adjusted to 5–6 with dilute hydrochloric acid. Ethanol was removed in vacuo. The resulting residue was purified by column chromatography eluting with petroleum ether and ethyl acetate (4:1) to afford compound 3 as a colorless oil (0.8 mg, 34% yield). MS (ESI): m/z 539.2 [M+H]+. 1H-NMR (CDCl3, 300 MHz) δ: 7.13 (t, J=6.1 Hz, 3H), 5.44–5.73 (m, 2H), 5.28 (dd, J=10.5, 3.3 Hz, 1H), 4.94–5.16 (m, 4H), 2.93–3.31 (m, 6H), 2.62 (s, 3H), 2.49 (s, 6H), 2.30 (s, 6H), 2.05 (s, 3H). 13C-NMR (CDCl3, 75 MHz) δ: 170.0, 168.3, 168.2, 167.6, 149.2, 147.2, 147.1, 141.9, 141.0, 135.2, 132.3, 132.2, 127.3, 124.3, 123.4, 119.3, 119.1, 88.0, 77.2, 70.5, 35.9, 27.7.

Fig. 6. Effects of Compound 14 on the Expression of Cytochrome c in t-BHP Treated H9c2 Cells
(A) Immunoblot and (B) densitometry analysis of the ratio of cytochrome c to β-actin. Data were presented as the mean±S.D. of three independent experiments.

*p<0.05 versus model group.

Fig. 7. Effects of Compound 14 on the Acute Myocardial Ischemia Model in Rats
Myocardial ischemia was induced by left anterior descending coronary artery occlusion in adult SD rats. Infarct size was presented as a percentage of the whole left ventricle area. Data were expressed as the mean±S.D., n=7 animals/group.

*p<0.05 versus model group. **p<0.01 versus model group.

%N, 14.10. Found: C, 60.14; H,7.20; N, 14.28.

4-(3,5,6-Trimethylpyrazin-2-yl)hepta-1,6-dien-4-ol (2) Compound 1 (2 g, 103 mmol) was dissolved in anhydrous THF (30 mL) and stirred at 0°C under N2 atmosphere for 0.5 h. Allylmagnesium bromide (1 M solution in diethyl ether, 23 mL) was added dropwise to the mixture. The reaction mixture was stirred at room temperature overnight. The reaction was quenched by saturated aqueous NH4Cl solution. The mixture was extracted with ethyl acetate (3×80 mL). The combined organics were washed with brine (30 mL), dried over Na2SO4 and concentrated under reduced pressure. The resulting residue was purified by column chromatography eluting with petroleum ether and ethyl acetate (6:1) to afford compound 2 as a yellow oil (0.8 mg, 34% yield). MS (ESI): m/z 233.0 [M+H]+, 255.6 [M+Na]+. 1H-NMR (300 MHz, DMSO-d6) δ: 2.40 (d, J=3.7 Hz, 6H, 2CH3), 2.54–2.71 (m, 7H, CH3, 2CH2), 4.92 (ddd, J=10.4, 2.4, 1.3 Hz, 4H, 2CH2), 5.41 (s, 1H, OH), 5.63 (ddt, J=17.3, 10.2, 7.1 Hz, 2H, 2CH). 13C-NMR (75 MHz, DMSO-d6) δ: 21.3, 21.5, 23.5, 44.6 (2C), 76.6, 117.9 (2C), 134.8 (2C), 146.3, 148.1, 148.8, 152.7. Anal. Calcd for C10H14N2O2·0.25H2O: C, 60.44; H, 7.35; N, 14.10. Found: C, 60.14; H,7.20; N, 14.28.

(R)-2-Acetoxy-3-(3,4-diacetoxyphenyl)propanoic Acid (3) To a solution of sodium Danshensu (10 g, 45 mmol) in acetic anhydride (40 mL) at room temperature was slowly added a catalytic amount of HClO4. The reaction mixture was stirred at room temperature for 3 h and then the mixture was poured into iced water. The mixture was extracted with ethyl acetate (4×100 mL). The combined organics were washed with water and dried over Na2SO4. Solvent was removed in vacuo. The resulting residue was purified by column chromatography eluting with petroleum ether and ethyl acetate (1:1) to afford compound 3 as an amorphous white solid (5.2 g, 36% yield).

(R)-4-(2-Acetoxy-3-chloro-3-oxopropyl)-1,2-phenylene Diacetate (4) To compound 3 (5.58 g, 17 mmol) in anhydrous CH2Cl2 (30 mL) was added oxalyl chloride (4.31 g, 34 mmol) dropwise and two drops of anhydrous DMF. The mixture was stirred at room temperature for 4 h. Removal of solvent gave a crude product of compound 4, which was used directly in the next step without further purification.

(R)-4-(2-Acetoxy-3-oxo-3-(4-(3,5,6-trimethylpyrazin-2-yl)hepta-1,6-dien-4-yl)oxy)propyl)-1,2-phenylene Diacetate (5) Compound 2 (2 g, 8.6 mmol) was dissolved in anhydrous THF (30 mL) and stirred at 0°C under N2 for 0.5 h. n-Butyllithium (1 M solution in diethyl ether, 23 mL) was added dropwise to the mixture. The mixture was stirred for 1 h. To the mixture was added a solution of compound 4 in anhydrous THF (10 mL). The mixture was warmed to room temperature and stirred until the reaction was completed as monitored by TLC. The pH of the mixture was adjusted to 5–6 with aqueous NaHCO3 solution and was then extracted with ethyl acetate (3×80 mL). The combined organics were washed with water and dried over Na2SO4 and filtered. Solvent was removed in vacuo. The resulting residue was purified by column chromatography eluting with petroleum ether and ethyl acetate (3:1) to afford compound 5 as a yellow oil (1.9 g, 41% yield). MS (ESI): m/z 539.2 [M+H]+. 1H-NMR (CDCl3, 300 MHz) δ: 7.13 (t, J=6.1 Hz, 3H), 5.44–5.73 (m, 2H), 5.28 (dd, J=10.5, 3.3 Hz, 1H), 4.94–5.16 (m, 4H), 2.93–3.31 (m, 6H), 2.62 (s, 3H), 2.49 (s, 6H), 2.30 (s, 6H), 2.05 (s, 3H). 13C-NMR (CDCl3, 75 MHz) δ: 170.0, 168.3, 168.2, 167.6, 149.2, 147.2, 147.1, 141.9, 141.0, 135.2, 132.3, 132.2, 127.3, 124.3, 123.4, 119.3, 119.1, 88.0, 77.2.
4-(3,5,6-Trimethylpyrazin-2-yl)hepta-1,6-dien-4-yl(3,4-dihydroxyphenyl)propoanato (6) To compound 5 (370 mg, 0.69 mmol) in a mixture of methanol and water (10:5 mL) was added Na2CO3 (146 mg, 1.38 mmol). The reaction mixture was stirred until the reaction was completed as monitored by TLC. Methanol was removed under reduced pressure. The reaction mixture was stirred at room temperature until the reaction was completely transformed as monitored by TLC. Solvent was completely transformed as monitored by TLC. Solvent was removed in vacuo. The residue was washed with diethyl ether and concentrated to afford compound 6 as a yellow oil (266 mg, 85% yield). MS (ESI): m/z 455.21 [M+H]+.

1H-NMR (300 MHz, dimethyl sulfoxide (DMSO)) δ: 8.80 (m, 2H), 6.71–6.58 (m, 2H), 6.56–6.42 (m, 1H, CH), 5.75–5.40 (m, 2H), 5.10 (ddd, J = 21.5, 14.4, 7.7 Hz, 5H), 3.08–2.89 (m, 5H), 2.79 (dd, J = 14.5, 10.1 Hz, 1H), 2.51 (s, 3H), 2.42 (s, 6H), 1.99 (s, 3H). 13C-NMR (75 MHz, CDCl3) δ: 170.5, 168.1, 150.2, 148.9, 148.0, 146.9, 144.2, 143.3, 132.2, 128.4, 128.4, 121.4, 119.3 (2C), 116.2, 115.2, 87.8, 80.2 (60.2), 77.2, 72.2, 42.4 (2C), 40.3, 40.0, 36.8, 28.3 (6C), 22.7, 21.5, 21.3, 20.5. Anal. Calcd for C37H50N4O11: C, 60.93; H, 6.82; N, 7.29. Found: C, 60.66; H, 6.72; N, 6.91.

Di-tert-butyl-3,3′-(((4-(2-hydroxy-3-oxo-3-((4-(3,5,6-trimethylpyrazin-2-yl)hepta-1,6-dien-4-yl)oxy)propyl)-1,2-phenylene)bis(oxo))bis(azanediyl))(R)-bis(2-oxopropanoate) (9) Compound 9 was prepared using a similar method to that as described for the synthesis of compound 8. Colorless oil, 89% yield. MS (ESI): m/z 727.15 [M+H]+. 1H-NMR (300 MHz, CDCl3) δ: 7.13 (d, J = 12.0 Hz, 3H), 5.70 (s, 3H), 5.10–4.96 (m, 4H), 4.09 (t, J = 6.2 Hz, 4H), 3.13 (dd, J = 14.5, 8.9 Hz, 6H), 2.53–2.39 (m, 9H), 1.44 (s, 18H). 13C-NMR (75 MHz, CDCl3) δ: 172.3, 171.2, 168.2 (2C), 156.3, 149.7, 149.2, 147.5, 146.6, 141.6, 140.5, 136.8, 132.3, 132.0, 127.9, 124.1, 123.0, 119.3 (2C), 87.7, 80.1, 71.2, 60.4, 42.3 (2C), 40.4, 40.0 (2C), 28.3 (6C), 22.5, 21.5, 21.1. HR-MS (ESI): [M+H]+ m/z 727.3517 (Calcd for C37H50N4O11: 727.3549).

(R)-4-(2-Acetoxy-3-oxo-3-((4-(3,5,6-trimethylpyrazin-2-yl)hepta-1,6-dien-4-yl)oxy)propyl)-1,2-phenylenebis(2-aminoacetate) (10) To compound 8 (100 mg, 0.13 mmol) in CH2Cl2 (2mL) at 0°C was added 2mL of TFA dropwise. The mixture was stirred at room temperature until compound 8 was completely transformed as monitored by TLC. Solvent was removed in vacuo. The residue was washed with diethyl ether and concentrated to afford compound 10 as a white solid (24mg, 68% yield). MS (ESI): m/z 569.41 [M+H]+. 1H-NMR (300 MHz, DMSO-D6) δ: 8.77 (d, J = 37.2 Hz, 6H), 7.38–7.24 (m, 3H), 7.57–5.43 (m, 2H), 5.33 (dd, J = 10.0, 3.8 Hz, 1H), 5.12–4.98 (m, 4H), 4.23 (d, J = 4.8 Hz, 4H), 3.34–2.77 (m, 6H), 2.54 (d, J = 10.0 Hz, 3H), 2.00 (d, J = 7.1 Hz, 6H), 2.00 (d, J = 5.9 Hz, 3H). HR-MS (ESI): [M+H]+ m/z 569.2601 (Calcd for C37H50N4O11: 569.2606).

(R)-4-(2-Hydroxy-3-oxo-3-((4-(3,5,6-trimethylpyrazin-2-yl)hepta-1,6-dien-4-yl)oxy)propyl)-1,2-phenylenebis(2-aminoacetate) (11) Compound 11 was prepared using a similar method as that described for the synthesis of compound 10. White solid (43mg, 74% yield). MS (ESI): m/z 525.15 [M−H]−. 1H-NMR (300 MHz, DMSO-D6) δ: 8.70 (s, 6H), 7.46–7.16 (m, 2H), 6.94 (dt, J = 20.3, 8.0 Hz, 1H), 5.79–5.38 (m, 3H), 5.03 (t, J = 11.0 Hz, 4H), 4.40–4.14 (m, 4H), 3.20–2.71 (m, 6H), 2.48 (s, 3H), 2.42 (d, J = 3.7 Hz, 6H). 13C-NMR (75 MHz, DMSO) δ: 172.0, 166.5, 166.4, 159.4, 159.0, 150.1, 149.3, 147.0, 140.9, 139.8, 138.5, 133.0, 132.7, 124.5, 123.4, 119.6, 88.8, 85.8, 71.3, 40.7 (2C), 39.8, 39.2 (2C), 22.7, 21.6, 21.3. HR-MS (ESI): [M+H]+ m/z 527.2493 (Calcd for C27H34N4O7: 527.2500).

(R)-4-(2-Hydroxy-3-oxo-3-((4-(3,5,6-trimethylpyrazin-2-yl)hepta-1,6-dien-4-yl)oxy)propyl)-1,2-phenylene Dicarboxylate (12) To compound 6 (200 mg, 0.44 mmol) in anhydrous CH2Cl2 (15mL) on an ice-water bath was added acetic anhydride (0.09 mL, 0.96 mmol) and triethylamine (97 mg, 0.96 mmol). The reaction mixture was stirred at room temperature until the reaction was completed as monitored by TLC. The mixture was washed with water (3×10 mL) and the combined organics were dried over Na2SO4. Solvent was removed in vacuo. The resulting residue was purified by column chromatography eluting with petroleum ether and ethyl acetate (3:1) to afford compound 8 as a colorless oil (209 mg, 85% yield). MS (ESI): m/z 119.3 (2C), 116.2, 115.2, 87.8, 80.2 (60.2), 77.2, 72.2, 42.4 (2C), 40.3, 40.0, 36.8, 28.3 (6C), 22.7, 21.5, 21.3, 20.5. Anal. Calcd for C2H5N2O2: C, 60.93; H, 6.82; N, 7.29. Found: C, 60.66; H, 6.72; N, 6.91.
H9c2 cells were harvested in a RIPA lysis buffer containing 1% phenylmethylsulfonyl fluoride (PMSF) and 1% protease inhibitor cocktail 4 h after t-BHP treatment. Cell lysis buffer were centrifuged at 13000×g for 10 min at 4°C and the supernatant was collected. The protein solution was quantified using the Pierce BCA Protein Assay Kit (Thermo) with bovine serum albumin as a standard control. The supernatant was mixed with 5× loading sample buffer and the mixture was boiled for 5 min at 100°C and stored at −20°C. The proteins were separated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were blocked at room temperature for 2 h and incubated with primary antibodies (1:1000) at 4°C overnight. Horseradish peroxidase-conjugated anti-rabbit antibody (1:2000) was added and incubated for 2 h at room temperature. Then, signals were obtained and the blots were washed and subsequently developed with an ECL Western blotting Detection Kit (GE Healthcare, U.K.) and photographs of protein blots were taken with a Molecular Imager (Carestream, Rochester, NY, U.S.A.).

### Protective Effect in a Rat Model of Acute Myocardial Ischemia

The protective effect of compound 14 was further studied in a rat model of acute myocardial ischemia. The rat model of myocardial ischemia was established by the ligation of left anterior descending (LAD) coronary artery for 2 h. All protocols were performed in accordance with guidelines for the Care and Use of Laboratory Animals of Jinan University. Briefly, after being anesthetized with pentobarbital sodium intraperitoneally (5%, w/v), male Sprague–Dawley (SD) rats weighing 250–270 g were ventilated with a positive pressure respirator at a stroke volume of 12 mL/kg and a rate of 60 strokes per minute with 95% O₂ and 5% CO₂ throughout the experiment. The rat heart was exposed through a left thoracotomy and the LAD was ligated 2–3 mm from its origin assay. Briefly, H9c2 cells were cultured in high glucose Dulbecco's modified Eagle's medium (DMEM) medium (Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco) and 100 units/mL penicillin–streptomycin (Gibco). Cells were seeded in 96-well plates at a density of 1×10⁴/well and incubated in a humidified atmosphere (37°C, 5% CO₂) for 24 h. After discarding the medium, cells were pretreated with compounds with indicated concentrations (the final concentrations were 0, 10, 30, 100 μM) for 1 h in serum free medium, and then exposed to 150 μM t-BHP for 12 h. MTT solution (0.5 mg/mL) was added and incubated for another 4 h. The supernatant was removed and DMSO (100 μL) was added subsequently. After dissolved completely, absorbance was read at a wavelength of 490 nm with a microplate reader (BioTek, America). Cell viability was expressed as the percentage with respect to the control cells.

#### Determination of Cell Apoptosis

H9c2 cells were grown in 35-mm dishes (1×10⁵ cells for each dish) and cultured for 24 h. Cells were then pretreated with different concentrations of compound 14 for 1 h, followed by exposure to t-BHP (150 μM) for 3 h. Cells were washed with ice-cold phosphate buffered saline (PBS) and fixed with 4% formaldehyde in PBS, and then incubated with 5 μM Hoechst at 37°C for 10 min. Nuclei were visualized at the same exposure time using a fluorescence microscope (Olympus Corporation, Japan).

#### Western Blot

H9c2 cells were harvested in a RIPA lysis buffer containing 1% phenylmethylsulfonyl fluoride (PMSF) and 1% protease inhibitor cocktail 4 h after t-BHP treatment. Cell lysis buffer were centrifuged at 13000×g for 10 min at 4°C and the supernatant was collected. The protein solution was quantified using the Pierce BCA Protei Assay Kit (Thermo) with bovine serum albumin as a standard control. The supernatant was mixed with 5× loading sample buffer and the mixture was boiled for 5 min at 100°C and stored at −20°C. The proteins were separated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were blocked at room temperature for 2 h and incubated with primary antibodies (1:1000) at 4°C overnight. Horseradish peroxidase-conjugated anti-rabbit antibody (1:2000) was added and incubated for 2 h at room temperature. Then, signals were obtained and the blots were washed and subsequently developed with an ECL Western blotting Detection Kit (GE Healthcare, U.K.) and photographs of protein blots were taken with a Molecular Imager (Carestream, Rochester, NY, U.S.A.).

#### Protective Effect on t-BHP-Induced Cell Damage in H9c2 Cells

The protective effects of new compounds in H9c2 cells subjected to t-BHP were measured by MTT assay. Briefly, H9c2 cells were cultured in high glucose Dulbecco's modified Eagle's medium (DMEM) medium (Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco) and 100 units/mL penicillin–streptomycin (Gibco). Cells were seeded in 96-well plates at a density of 1×10⁴/well and incubated in a humidified atmosphere (37°C, 5% CO₂) for 24 h. After discarding the medium, cells were pretreated with compounds with indicated concentrations (the final concentrations were 0, 10, 30, 100 μM) for 1 h in serum free medium, and then exposed to 150 μM t-BHP for 12 h. MTT solution (0.5 mg/mL) was added and incubated for another 4 h. The supernatant was removed and DMSO (100 μL) was added subsequently. After dissolved completely, absorbance was read at a wavelength of 490 nm with a microplate reader (BioTek, America). Cell viability was expressed as the percentage with respect to the control cells.

### Protective Effect in a Rat Model of Acute Myocardial Ischemia

The protective effect of compound 14 was further studied in a rat model of acute myocardial ischemia. The rat model of myocardial ischemia was established by the ligation of left anterior descending (LAD) coronary artery for 2 h. All protocols were performed in accordance with guidelines for the Care and Use of Laboratory Animals of Jinan University. Briefly, after being anesthetized with pentobarbital sodium intraperitoneally (5%, w/v), male Sprague–Dawley (SD) rats weighing 250–270 g were ventilated with a positive pressure respirator at a stroke volume of 12 mL/kg and a rate of 60 strokes per minute with 95% O₂ and 5% CO₂ throughout the experiment. The rat heart was exposed through a left thoracotomy and the LAD was ligated 2–3 mm from its origin.
between the pulmonary conus and left atrium with a 5-0 silk suture. Rats in sham-operated group were subjected to the entire surgical procedure but without LAD ligation. After surgery, the chest was closed. Ten minutes after LAD ligation, rats in drug-treated groups received an i.v. injection of different drugs with indicated doses. The rats in sham-operated and model groups received a same volume of saline. Twenty-four hours after drug administration, rats were re-anesthetized. The rat heart was isolated and then frozen at −20°C for 30 min. The atrium, arteries and pericardium were removed. The left ventricle was sectioned into 6 slices and then incubated in 2% (w/v) 2,3,5-triphenyl-tetrazolium chloride (TTC) solution at 37°C for 10 min. The heart slices were photographed with a digital camera to distinguish the red-stained viable tissues and the white-unstained necrotic tissues. Areas of infarct size were measured digitally using Image J software. The infarct size was expressed as a percentage of the total left ventricular area.

Conclusion
Both DSS and TMP are widely used to treat cardiovascular diseases in China. However, sub-optimal efficacy limited their clinical benefits. To improve their efficacy, we have designed and synthesized numerous DSS–TMP conjugates. One of the compounds, i.e., ADTM, showed much improved therapeutic efficacy than both DSS and TMP. However, unfortunately, ADTM suffers from two problems. One is poor water solubility, and the other short half-life due to cleavage of the ester bond between DSS and TMP. The new compound 14, not only have improved therapeutic efficacy, also have good water solubility and improved stability. In summary, compound 14 is a promising new drug candidate for the treatment of myocardial ischemia.

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Conflict of Interest The authors declare no conflict of interest.

References
1) Connolly M., Menown I. M., Adv. Ther., 30, 369–386 (2013).
2) Zhao Z. Q., Curr. Opin. Pharmacol., 4, 159–165 (2004).
3) Zweier J. L., Talukder M. A., Circ. Res., 70, 181–190 (2006).
4) Singal P., Li T., Kumar D., Danelisen I., Iliiskovic N., Mol. Cell. Biochem., 207, 77–86 (2000).
5) Haustetter A., Izumo S., Circ. Res., 82, 1111–1129 (1998).
6) Buja L. M., Entman M. L., Circulation, 98, 1355–1357 (1998).
7) Zhou L., Zuo Z., Chow M. S., J. Clin. Pharmacol., 45, 1345–1359 (2005).
8) Hu P., Luo G. A., Zhao Z. Z., Zhang J. H., Chem. Pharm. Bull., 53, 481–486 (2005).
9) Cui G., Shan L., Hung M., Lei S., Choi I., Zhang Z., Yu P., Hoi P., Wang Y., Lee S. M. Y., Int. J. Cardiol., 168, 1349–1359 (2013).
10) Cao C. M., Xia Q., Zhang X., Xu W. H., Jiang H. D., Chen J. Z., Life Sci., 72, 2451–2463 (2003).
11) Jia Y., Dong X., Zhou P., Liu X., Pan L., Xin H., Zhu Y. Z., Wang Y., Eur. J. Med. Chem., 55, 176–187 (2012).
12) Wu L., Qiao H., Li Y., Li L., Phytomedicine, 14, 652–658 (2007).
13) Zhang L., Chen L., Lu Y., Wu J., Xu B., Sun Z., Zheng S., Wang A., Eur. J. Pharmacol., 643, 195–201 (2010).
14) Su X. H., Liang D. Q., Wang X. M., Chinese J. Pathophysiol., 8, 122–124 (1992).
15) Shyu K. G., Wang M. T., Wang B. W., Chang C. C., Lee J. G., Kuan P., Chang H., Cardiovasc. Res., 54, 576–583 (2002).
16) Cui Q., Chen Y., Zhang M., Shan L., Sun Y., Yu P., Zhang G., Wang D., Zhao Z., Xu Q., Xu B., Wang Y., Chem. Biol. Drug Des., 84, 282–291 (2014).
17) Xiao X., Liu Y., Qi C., Qiu F., Chen X., Zhang J., Yang P., Neurol. Res., 32, 547–555 (2010).
18) Li S., Shan L. C., Zhang Z. J., Li W., Liao K. Y., Li S., Sheng X. Y., Yu P., Wang Y. Q., J. Chromatogr. Sci., 53, 872–878 (2015).
19) Sun Y. W., Tan Z. C., Liang Z. B., Wang L., Shan L. C., Yu P., Lee S. M., Wang Y. Q., MedChemComm, 6, 586–591 (2015).
20) Xu C., Deng H., Chen H., Cui Q., Shan L., Yu P., Sun Y., Wang Y., Asian J. Chem., 28, 2555–2561 (2016).
21) Cuilio A. G., Kuppusamy C. P., DiPaula A., Am. J. Physiol. Heart Circ. Physiol., 280, 2649–2657 (2001).
22) Grech E. D., Dodd N. J. F., Jackson M. J., Morrisson W. L., Faragher E. B., Ramsdale D. R., Am. J. Cardiol., 77, 122–127 (1996).
23) Dhalia N. S., Elmosilhi A. B., Hata T., Makino N., Cardiovasc. Res., 47, 446–456 (2000).
24) Heitzer T., Schlizig T., Krohn K., Meinertz T., Münzel T., Circulation, 104, 2673–2678 (2001).
25) Myers M. L., Bolli R., Lekich R. F., Hartley C. J., Roberts R., Circulation, 72, 915–921 (1985).
26) Li W., Pi R., Chan H. H., Fu H., Lee N. T., Tsang H. W., Pu Y., Chan D. C., Li C., Luo J., Xiong K., Li Z., Xue H., Carlier P. R., Pang Y., Tsim K. W., Li M., Han Y., J. Biol. Chem., 280, 1–38 (2005).
27) Tsutsui H., Kinugawa S., Matsushima S., Cardiovasc. Res., 81, 449–456 (2009).
28) Zimmermann A. K., Loucks F. A., Schroeder E. K., Bouchard R. J., Tyler K. L., Linseman D. A., J. Biol. Chem., 282, 29296–29304 (2007).
29) Almenri E. S., Livingstone D. J., Nicholson D. W., Salvesen G., Thornberry N. A., Wong W. W., Yuan J., Cell, 87, 171 (1996).