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Design, Synthesis and Biological Evaluations of Novel Conjugates of Danshensu, Tetramethylpyrazine and Hydrogen Sulfide Donors as Cardioprotective Agents

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A series of molecular hybrids of Danshensu-tetramethylpyrazine-hydrogen sulfide donors have been synthesized and evaluated *in vitro* as potent antimyocardial ischemia agents. The pharmacological data demonstrated that compound DTA-2 exhibited the most potent protective activities against *tert*-butyl hydroperoxide (*t*-BHP) induced injury in H9c2 cells. Further study showed that DTA-2 exhibited better ROS-scavenging activity and mitochondria protective effect than its parental drug DTM, ACS48 and mixtures of these two. These results indicated that DTA-2 may work through a unique mechanism and may serve as a drug candidate for anti-myocardial ischemia therapy that require further study.

Keywords: Danshensu, Tetramethylpyrazine, H_2S donor, Synergistic effect, Myocardial ischemia/reperfusion injury.

INTRODUCTION

Ischemic heart disease caused by the abnormal function of heart and blood vessels is a leading cause of death and morbidity worldwide [1]. Current treatments for myocardial ischemia mainly focus on the recovery of the blood flow to ischemic heart *via* revascularization [2]. A number of thrombolytic and antiplatelet agents have shown great therapeutic benefits for the treatment of myocardial ischemia [3]. However, the therapeutic benefits of single use of thrombolytic agents aiming at the revascularization of blood vessels were quite limited partially because of the overproduction of free radicals in the process of reperfusion. The over production of free radicals leads to an extensive damage of cellular components, including mitochondrial dysfunction which will cause cell apoptosis [4]. Thus, the inhibition of cellular oxidative injury induced by free radicals would provide cardioprotective effects. In fact, increasing evidence demonstrated that antioxidants exhibited cardioprotective effects in cultured cells and rat models of myocardial ischemia [5,6].

In China, many traditional herbs are used to prevent and treat myocardial ischemia. Among them, *Salvia miltiorrhiza* (Danshen) and *Ligation wallachia Franchat* (Chuanxiong) have been used for many years [7,8]. As the major active ingredient, danshensu 3-(3,4-dihydroxyphenyl)lactic acid (DSS, Fig. 1), isolated from danshen and tetramethylpyrazine (TMP, Fig. 1), isolated from Chuanxiong have a variety of biological activities, which include dilating coronary arteries, inhibiting platelet aggregation, scavenging free radicals, improving microcirculation and anti-inflammatory properties [9,10]. Both of them have been used in clinic for the treatment of heart disease. However, their therapeutic benefits are limited due to weak activities.

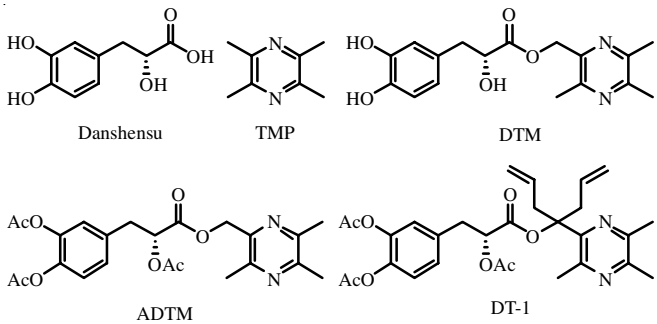


Fig. 1. Structures of DSS, TMP and related compounds

To enhance the therapeutic effects of them, a series derivative of DSS and TMP were synthesized to acquire additive effects of them and improve their pharmacokinetic properties. We discovered a compound named ADTM (Fig. 1), exhibiting much better activity than DSS and salvianolic acid B (SAB) *in vitro* and *in vivo* [11,12]. The DT-1 (Fig. 1) was synthesized to obtain a prolonged half-life, which exhibited a longer and stronger protective effect than ADTM *in vitro* [13].

Hydrogen sulfide (H_2S) is an essential body product, known as the third cellular signaling molecule besides NO and CO [14,15]. It is a powerful reducing agent with various endogenous biological actions, including vasorelaxing and cardioprotective effects [16]. The cardioprotective effects of H_2S may be attributed to its anti-apoptotic, anti-inflammatory and antioxidative effects [17,18]. Recently, H_2S donors have been intensively studied *in vitro* and *in vivo* and some of them displayed great therapeutic potential for the treatment of myocardial ischemia [19,20].

Given that our synthesized DSS-TMP conjugates and H_2S both hold protective effects on cardiovascular system and based on medicinal chemical hybridization (MCH) strategy [21,22], we coupled the DTTs (H_2S donor) with our synthesized DSS-TMP conjugates to determine if it could provide a new kind of hybrids exhibited a potent and synergistic antimyocardial ischemia/reperfusion activity.

EXPERIMENTAL

All commercially available chemicals were of reagent grade and used directly without further purification unless otherwise noted. Reactions were magnetically stirred and monitored by thin layer chromatography (TLC) with 0.25 mm precoated silica gel plates. ^{1}H NMR/^{13}C NMR spectra were recorded at ambient temperature on a Bruker 300 MHz spectrometer in $CDCl_3$ or DMSO-d_6. Electrospray ionization mass spectra (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 mass spectra (HRMS) were obtained on a Waters Vevo G2 Q-Tof mass spectrometer by electrospray ionization.

ACS5 was prepared according to a published method of Pittman *et al.* [23], ACS48, ACS50 was prepared according to a published method reported by Lee *et al*. [24].

Preparation of compound DTA-1- 9

Preparation of compound DTA-1,3,5,6,7,8,9: To a mixture of anhydrous CH_2Cl_2 (3 mL) and DMF (0.5 mL) was added ACS48, ACS5 or ACS50 (0.5 mmol) and the mixture was stirred until DTT was completely dissolved. To the mixture was added 1-ethyl-3-(3-dimethyl aminopropyl)carbodiimide hydrochloride (EDCI, 0.5 mmol), 4-(dimethylamino)pyridine (DMAP, 0.4 mmol) and a solution of BDTM or DT-2 (0.2 mmol) in CH_2Cl_2 (0.5 mL). The mixture was stirred at room temperature for 1.5 h under a nitrogen atmosphere. The organic layer was collected and washed with water (3 mL × 2) and then by saturated NaCl solution (3 mL × 2). The combined organic layers were dried over anhydrous Na_2SO_4. Solvents were removed *in vacuo* and the residue was purified by flash chromatography to provide compounds DTA-1,3,5,7,8,9. In the synthesis process of DTA-7 except change the amount of DMAP, EDCI to (EDCI, 1 mmol), (DMAP, 1.2 mmol), the other was same as stated above.

(R)-4-(3-Oxo-2-((4-(3-thioxo-3H-1,2-dithiol-4-yl)-benzoyl)oxy)-3-((3,5,6-trimethylpyrazin-2-yl) methoxy)-propyl)-1,2-phenylene diacetate (DTA-1): Brick red oil; yield 50 %; ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.47 (s, 1H, CH), 8.08-8.05 (m, 2H, arom.), 7.66-7.60 (m, 2H, arom.), 7.21-7.08 (m, 3H, arom.), 5.53-5.49 (dd, *J* = 8.2, 4.6 Hz, 1H, CH), 5.31-5.28 (m, 2H, CH_2), 3.36-3.31 (m, 2H, CH_2), 2.53-2.48 (m, 9H, 3×CH_3), 2.28 (m, 6H, 2×CH_3); ^{13}C NMR (75 MHz, $CDCl_3$): δ 213.39, 168.94, 168.15, 168.06, 165.26, 154.62, 151.46, 149.01 (2C), 148.17, 143.96, 142.01, 141.17, 138.13, 134.65, 129.97 (2C), 129.44, 129.11 (2C), 127.33, 124.53, 123.45, 73.10, 65.81, 36.79, 21.68, 21.39, 20.63(2C), 20.37. MS *m/z*: 653.0 $[M+H]^+$. HRMS (ESI) *m/z* calculated for $C_{31}H_{29}N_2O_8S_3$ $[M+H]^+$: 653.1086, found: 653.1087.

(R)-4-(3-Oxo-2-((4-(3-thioxo-3H-1,2-dithiol-4-yl)-benzoyl)oxy)-3-((4-(3,5,6-trimethylpyrazin-2-yl)hepta-1,6-dien-4-yl)oxy)propyl)-1,2-phenylene diacetate (DTA-3): Brick red semisolid; yield 41 %; ^{1}H NMR (300 MHz, DMSO-d_6): δ 9.25 (s, 1H, CH), 8.00-7.96 (m, 2H, arom.), 7.79-7.71 (m, 2H, arom.), 7.36-7.32 (t, 2H, arom.), 7.22-7.20 (d, *J* = 6 Hz, 1H, arom.), 5.75-5.48 (m, 3H, 3×CH), 5.10-5.01 (m, 4H, 2×CH_2), 3.44-2.91 (m, 6H, 3×CH_2), 2.59 (s, 3H, CH_3), 2.42 (s, 6H, 2×CH_3), 2.28-2.20 (m, 6H, 2×CH_3); ^{13}C NMR (75 MHz, DMSO-d_6): δ 213.84, 168.65, 168.61, 167.45, 165.03, 160.90, 149.70, 149.34,147.19, 147.16, 147.13, 142.22, 141.32, 139.14, 135.54, 132.81, 132.41, 129.75, 129.68,128.83, 127.90, 124.96, 123.90, 119.90, 87.32, 72.97, 36.41, 22.71, 21.62, 21.39, 20.79 (2C); MS *m/z*: 733.1 $[M+H]^+$; HRMS (EI) *m/z* calculated for $C_{37}H_{37}N_2O_8S_3$ $[M+H]^+$: 733.1712, found: 733.1707.

(R)-4-(3-Oxo-2-((2-thioxo-1,3-dithiol-4-carbonyl)-oxy)-3-((3,5,6-trimethylpyrazin-2-yl) methoxy)propyl)-1,2-phenylene diacetate (DTA-5): Pale yellow oil; yield 43 %; ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.95 (s, 1H), 7.11 (m, 3H), 5.36-5.30 (m, 3H), 3.30-3.21 (m, 2H), 2.52-2.48 (t, 9H), 2.28 (s, 6H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 211.25, 168.18 (2C), 167.92 (2C), 156.61, 151.66, 149.14, 148.77, 143.62, 142.04, 141.33, 139.82, 134.12, 133.82, 127.21, 124.75, 123.65, 73.99, 65.90, 36.51, 21.71, 21.42, 20.69, 20.61, 20.35; MS *m/z*: 577.0 $[M+H]^+$; HRMS (EI) *m/z* calculated for $C_{25}H_{25}N_2O_8S_3$ $[M+H]^+$: 577.0768, found: 577.0763.

(R)-4-(3-Oxo-2-((2-thioxo-1,3-dithiol-4-carbonyl)-oxy)-3-((4-(3,5,6-trimethylpyrazin-2-yl)hepta-1,6-dien-4-yl)oxy)propyl)-1,2-phenylene diacetate (DTA-6): Pale yellow semisolid; yield 36 %; ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.94 (s, 1H), 7.19-7.12 (m, 3H), 5.71-5.46 (m, 2H), 5.37-5.32 (dd, *J* = 10.5, 5.1 Hz, 1H), 5.11-5.04 (m, 4H), 3.36-3.21 (dd, *J* = 14.25, 3 Hz, 1H), 3.18-3.02 (m, 5H), 2.63 (s, 3H), 2.51-2.49 (d, *J* = 5 Hz, 6H), 2.31-2.30 (d, *J* = 1.2 Hz, 6H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 211.31, 168.24 (2C), 166.38 (2C), 156.46, 149.38, 149.27, 147.67, 146.71, 142.09, 141.31, 139.60, 134.22, 134.17, 132.05, 131.86, 127.23, 124.57, 123.74, 119.55, 119.47, 88.58, 73.92, 40.32, 40.00, 36.82, 22.57, 21.50, 21.20, 20.71, 20.65; MS *m/z*: 657 $[M+H]^+$; HRMS (EI) *m/z* calculated for $C_{31}H_{33}N_2O_8S_3$ $[M+H]^+$: 657.1399, found: 657.1404.

(R)-3-(3,4-Dihydroxyphenyl)-1-oxo-1-((4-(3,5,6-trimethylpyrazin-2-yl)hepta-1,6-diene-4-yl)oxy)propan-2-yl-2-thioxo-1,3-dithiol-4-carboxylate (DTA-7): Pale yellow oil; yield 35 %; ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.87 (s, 1H), 6.79-6.76 (m, 1H), 6.67-6.60 (m, 2H), 5.75-5.44 (m, 2H), 5.40-5.36 (dd, *J* = 10.5, 3 Hz, 1H), 5.14- 5.04 (m, 4H), 3.23-3.00 (m, 6H), 2.51-2.47 (d, 9H); ^{13}C NMR (75 MHz, $CDCl_3$): δ

211.30, 166.75 (2C), 156.59, 150.09, 150.01, 149.06, 148.24, 146.48, 144.29, 143.73, 139.21, 134.42, 132.08, 131.65, 127.53, 121.79, 119.56, 116.40, 115.23, 88.17, 74.48, 40.17, 40.03, 39.56, 36.60, 21.76, 21.48, 20.65. MS *m/z*: 573.1 $[M+H]^+$; HRMS (EI) *m/z* calculated for $C_{27}H_{29}N_2O_6S_3$ $[M+H]^+$: 573.1182, found: 573.1176.

(R)-4-(2-(2-(2-Methoxy-4-(3-thioxo-3H-1,2-dithiol-5-yl)phenoxy)acetoxy)-3-oxo-3-((3,5,6-trimethylpyrazin-2-yl)methoxy)propyl)-1,2-phenylene diacetate (DTA-8): Brown oil; yield 35 %; ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.42 (s, 1H), 7.20-7.17 (m, 1H), 7.11-7.05 (m, 4H), 6.68-6.65 (d, *J* = 9 Hz, 1H), 5.44-5.40 (dd, *J* = 9.6, 3.3 Hz, 1H), 5.31-5.28 (m, 2H), 4.82 (s, 2H), 3.91 (s, 3H), 3.36-3.21 (dd, *J* = 14.5, 3.6 Hz, 1H), 3.18-3.07 (m, 1H), 2.53-2.50 (m, 9H, 3×CH_3), 2.32-2.28 (m, 6H, 2×CH_3); ^{13}C NMR (75 MHz, $CDCl_3$): δ 215.12, 172.99, 168.36, 168.22, 167.41, 166.71, 150.38, 149.69, 142.03, 141.17, 135.17, 134.61, 132.10, 131.98, 127.10, 125.45, 124.38, 123.55, 120.52, 119.47, 119.38, 113.50, 110.07, 88.36, 72.66, 65.33, 56.18, 40.36, 40.10, 36.83, 22.74, 21.51, 21.31, 20.72; MS *m/z*: 713.1 $[M+H]^+$; HRMS (EI) *m/z* calculated for $C_{33}H_{33}N_2O_{10}S_3$ $[M+H]^+$: 713.1292, found: 713.1289.

(R)-4-(2-(2-(2-Methoxy-4-(3-thioxo-3H-1,2-dithiol-5-yl)phenoxy)acetoxy)-3-oxo-3-((4-(3,5,6-trimethylpyrazin-2-yl)hepta-1,6-diene-4-yl)oxy)propyl)-1,2-phenylene-diacetate (DTA-9): Brown semisolid; yield 37 %; ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.43 (s, 1H), 7.17-7.07 (m, 5H), 6.53-6.50 (d, 1H), 5.71-5.48 (m, 2H), 5.45-5.40 (dd, *J* = 11.4, 3.6 Hz, 1H), 5.10-5.04 (m, 4H), 4.77 (s, 2H), 3.89 (s, 3H), 3.37-3.31 (dd, *J* = 14.55, 3 Hz, 1H), 3.17-2.98 (m, 5H), 2.63-2.61 (m, 3H, CH_3), 2.49 (s, 6H, 2×CH_3), 2.34-2.30 (m, 6H, 2×CH_3); ^{13}C NMR (75 MHz, $CDCl_3$): δ 215.14, 172.86, 168.30, 168.24, 168.09, 167.64, 151.63, 150.48, 149.79, 149.13, 148.79, 143.69, 141.98, 141.20, 135.17, 134.18, 127.20, 125.57, 124.46, 123.43, 120.49, 113.82, 110.20, 72.93, 65.84, 65.49, 56.22, 36.47, 21.69, 21.40, 20.65, 20.37; MS *m/z*: 793.1 $[M+H]^+$; HRMS (EI) *m/z* calculated for $C_{39}H_{41}N_2O_{10}S_3$ $[M+H]^+$: 793.1923, found: 793.1912.

Preparation of compound DTA-2,4: To a solution of DTA-1 or DTA-3 (0.05 mmol) in CH_3OH (2 mL) was added H_2O (0.5 mL), followed by the adding of Na_2CO_3 (0.05 mmol). The mixture was stirred at room temperature for 10 min. Methanol was removed under vacuum. The water layer was then extracted with ethyl acetate (3 mL × 3). The combined organic layer was washed with saturated NaCl solution (3 mL × 2), dried over Na_2SO_4 and concentrated. The crude product was purified by column chromatography to afford DTA-2,4.

(R)-3-(3,4-Dihydroxyphenyl)-1-oxo-1-((3,5,6-trimethylpyrazin-2-yl)methoxy)propan-2-yl -4-(3-thioxo-3H-1,2-dithiol-4-yl)benzoate (DTA-2): Brick red oil; yield 75 %; ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.47 (s, 1H, CH), 8.06-8.03 (m, 2H, arom.), 7.64-7.56 (m, 2H, arom.), 6.77-6.62 (m, 3H, arom.), 5.46-5.43 (dd, *J* =6.9, 5.4 Hz, 1H, CH), 5.34-5.24 (m, 2H, CH_2), 3.25-3.1 (m, 2H, CH_2), 2.53-2.45 (m, 9H, 3×CH_3). ^{13}C NMR (75 MHz, DMSO): δ 213.83, 169.29, 165.25, 160.87, 151.50, 149.04, 148.93, 147.14, 145.52, 145.46, 144.65, 144.39, 139.11, 129.79, 129.62, 128.98, 126.94, 120.54, 117.15, 115.89, 74.06, 65.70, 36.66, 21.68, 21.39, 20.37; MS *m/z*: 569.0 $[M+H]^+$; HRMS (EI) *m/z* calculated for $C_{27}H_{25}N_2O_6S_3$ $[M+H]^+$: 569.0875, found: 569.0874.

(R)-3-(3,4-Dihydroxyphenyl)-1-oxo-1-((4-(3,5,6-trimethylpyrazin-2-yl)hepta-1,6-diene-4-yl)oxy)propan-2-yl 4-(3-thioxo-3H-1,2-dithiol-4-yl)benzoate (DTA-4): Brick red semisolid; yield 81 %; ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.43 (s, 1H), 8.03-8.01 (d, *J* = 8.4 Hz, 2H), 7.60-7.57 (m, 2H), 6.80-6.69 (dd, *J* = 27, 8.4 Hz, 3H), 5.72-5.45 (m, 3H), 5.11-5.01 (q, *J* = 10.5 Hz, 4H), 3.26-2.96 (m, 6H), 2.51-2.49 (m, 9H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 213.33, 167.87, 165.11, 154.85, 150.15, 150.07, 148.90, 148.05, 146.83, 146.75, 144.04, 143.54, 137.99, 132.25, 131.90, 129.82, 129.61, 129.05, 128.37, 121.87, 119.27, 116.41, 115.21, 87.80, 73.40, 60.47, 40.05, 29.32, 22.15, 21.44, 20.83; MS (ESI) *m/z*: 649.1 $[M+H]^+$; HRMS (EI) *m/z* calculated for $C_{33}H_{33}N_2O_6S_3$ $[M+H]^+$: 649.1495, found: 649.1494.

Biological evaluation

Protective effect on cultured cells: The protective effects of new compounds on H9c2 cells subjected to *t*-BHP were determined by the MTT assay[12]. Briefly, cells were seeded in 96-well plate at a density of 1×10^4/mL and cultured in high-glucose DMEM medium supplemented with 10 % fetal bovine serum and 100 units/mL penicillin-streptomycin. Cells were incubated in a humidified atmosphere (37 °C, 5 % CO_2) until grown to 90 % confluence. To evaluate the cardioprotective effects of the new compounds, cells were pretreated with different compounds at various concentrations (3, 10, 30, 100 μM). After incubation for 1 h, cells were then exposed to 150 μM *t*-BHP for another 12 h. MTT was added and the cells were incubated for another 4 h. Dimethyl sulphoxide was added and incubated for 0.5 h. Absorbance was read at a wavelength of 490 nm on a microplate reader. Cell viability was expressed as a percentage with respect to that of the untreated cells. SAB, DTM, ACS48 and the mixture of DTM and ACS48 were used as positive controls.

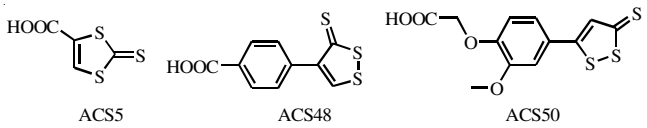
Free radical scavenging effect: The free radical scavenging effects of DTA-2 were detected according to a published method with minor modifications [25]. Briefly, cells were seeded in 96-well plate and pretreated with different drugs for 1 h. Cells were then incubated with CM-DCFHDA (10 μM) at 37 °C for 0.5 h in the dark, followed by treatment with *t*-BHP (150 μM) for another 1 h. The fluorescence intensity was measured at 480 nm excitation and 530 nm emissions using a fluorescence microplate reader (Bio-Rad Model 680, OEM System Co., Ltd., Kyoto, Japan). SAB, DTM, ACS48 and the mixture of DTM and ACS48 were used as positive controls.

Effect on mitochondrial membrane potential: The effect of DTA-2 on mitochondrial membrane potential was tested using a method previously reported [26]. JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl-benzimidazolyl- carbocyanine-iodide) was used as a molecular probe to measure mitochondrial membrane potential (Δψm). H9c2 cells were placed on 96-well at a density of 1×10^4/well and cultured in high-glucose DMEM medium supplemented with 10 % fetal bovine serum and 100 units/mL penicillin-streptomycin. Cells were pretreated with different drugs for 1 h, followed by incubation with 150 μM *t*-BHP for another 1 h. Cells were then washed with JC-1

buffer and stained with 2 μM JC-1 for 30 min. Fluorescence intensity was measured on a microplate reader at 488 nm excitation and 529 nm/590 nm dual emissions. The mitochondrial accumulation of JC-1 was dependent on Δψm and reflected by a shift in 529 nm and 590 nm emissions. Mitochondrial membrane depolarization was indicated by a decrease in the ratio of 590 nm to 529 nm emissions. SAB, DTM, ACS48 and the mixture of DTM and ACS48 were used as positive controls.

RESULTS AND DISCUSSION

The procedures for the synthesis of DTA-1-9 were described in **Schemes I** and **II**. The H_2S donors (ACS48, ACS50 and ACS5, Fig. 2) were synthesized according to the methods reported previously. BDTM and DT-2 were synthesized previously in our lab. BDTM or DT-2 was treated with different H_2S donors in the presence of 4-(dimethylamino)pyridine (DMAP) and 1-ethyl-3-(3-dimethyl aminopropyl)carbodiimide hydrochloride (EDCI) to afford the corresponding compounds DTA-1,3,5,6,7,8 and 9. DTA-1 and DTA-3 underwent hydrolysis in the presence of Na_2CO_3 to afford compounds DTA-2 and DTA-4, respectively.

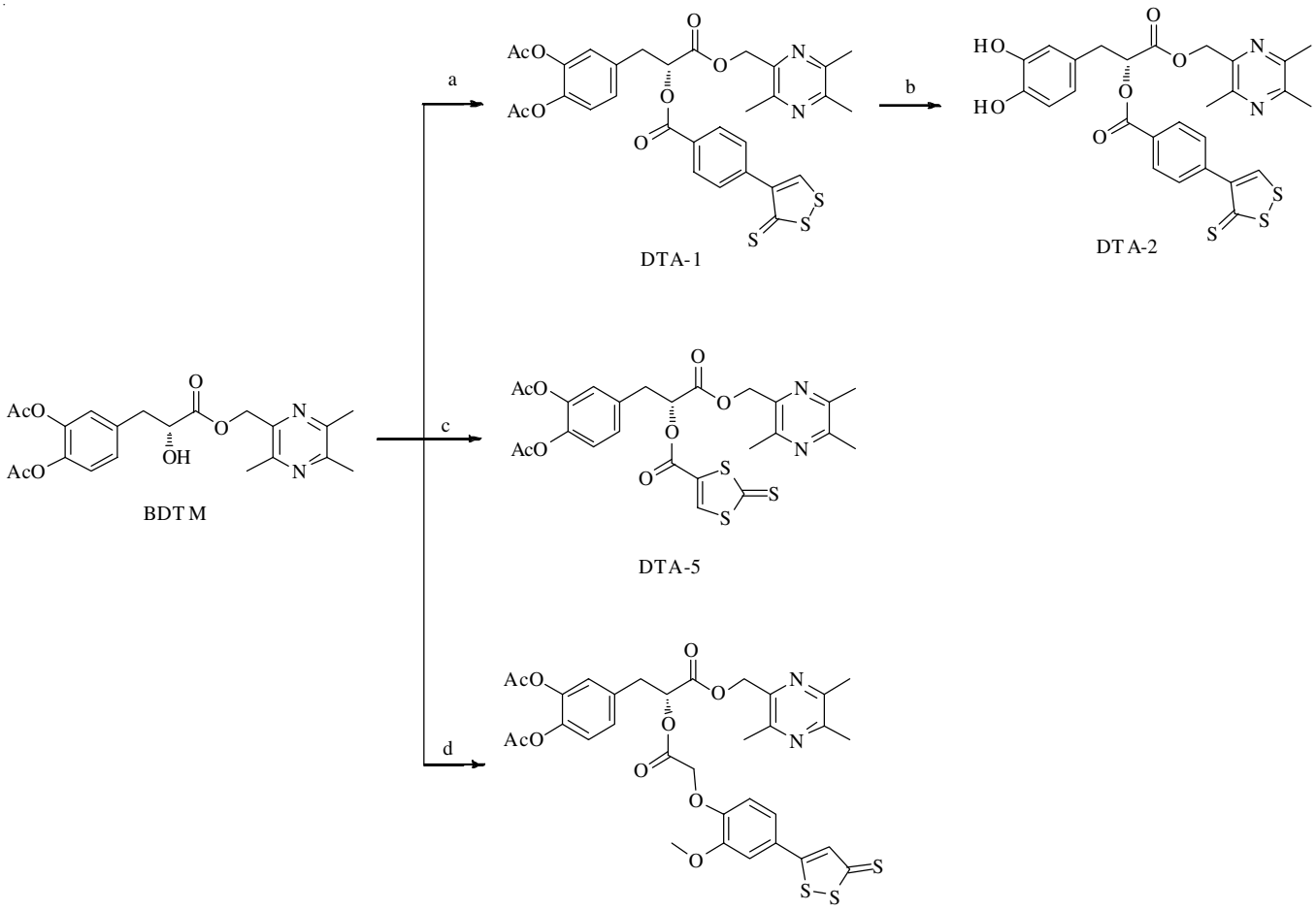


ACS5 ACS48 ACS50

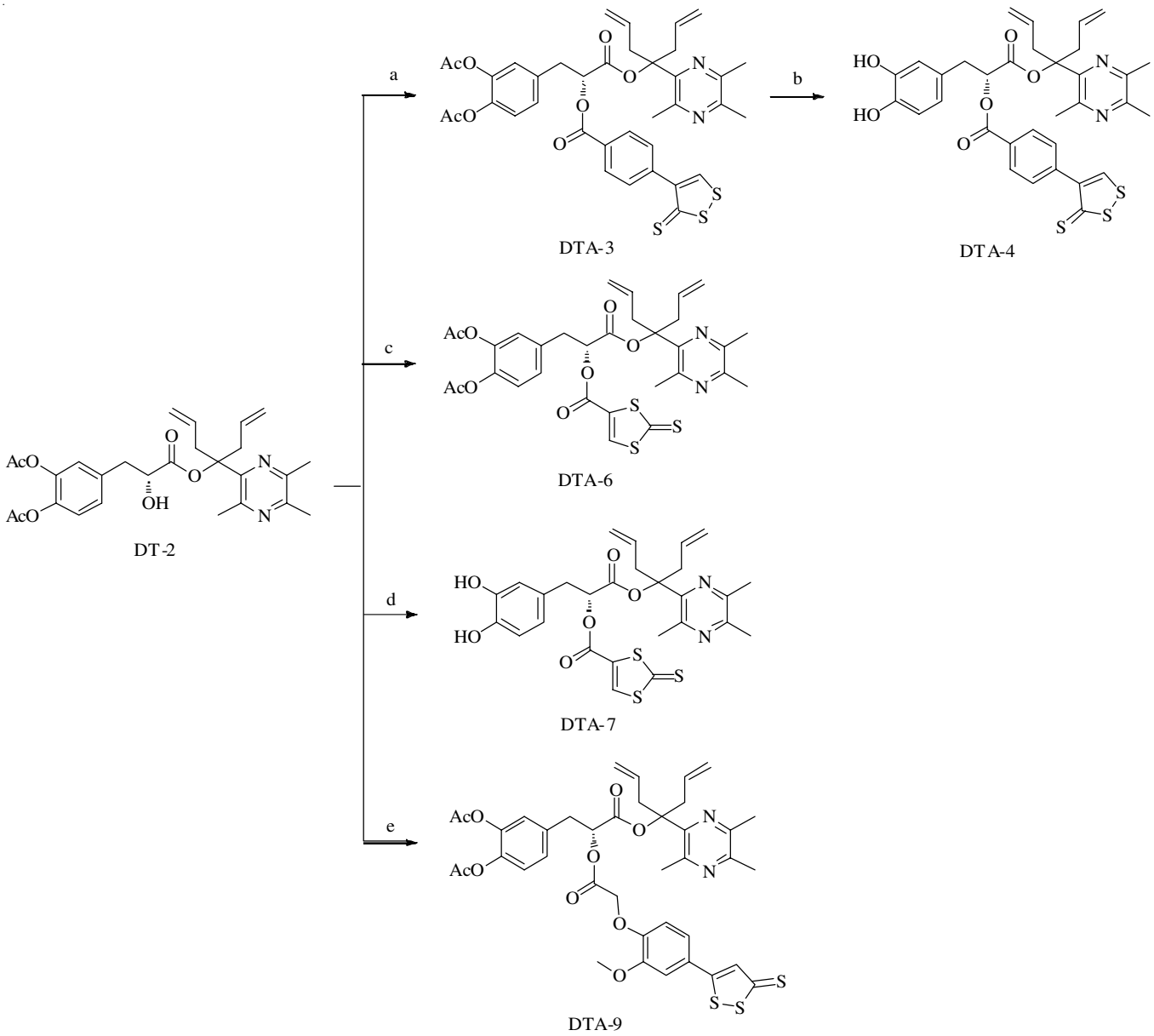
Fig. 2. Structures of the H_2S-releasing compounds

Biological activities: All the target compounds were first evaluated for their cardioprotective effects against *t*-BHP induced injury in H9c2 myocardial cells by the MTT assay [26]. Compounds with promising protective effects were chosen for further evaluation, including cardioprotective effect, free radical scavenging activity [25] and mitochondrial protective effect [25].

The protective effects of the target compounds against *t*-BHP induced injury in H9c2 cells were first studied. Salvianolic acid B (SAB), a clinically used agent for treatment of myocardial ischemia in China, was used as a positive control. E_{max} (%) (the percentage of maximum protection) is a parameter used to express the efficacy. As shown in Fig. 3, DTA-2 and DTA-4 significantly prevented cells against *t*-BHP induced injury compared with *t*-BHP treatment. DTA-1, DTA-3 and DTA-7-9 showed moderate activities in protecting cells. In sharp contrast, DTA-5-6 showed no activities in protecting cells. In analysis of the structure-activity relationship, we found that the protective effects of DTA-2, DTA-3 and DTA-4 beared an ACS48 moiety were stronger than other compounds with ACS5 or ACS50, indicating that ACS48 was better than ACS5 and ACS50 in enhancing the activities of DSS-TMP conjugates. Additionally, in comparison of the activities of DTA-1 with DTA-2, DTA-3 with DTA-4, or DTA-6 with DTA-7, the free phenolic hydroxyl groups were necessary for increasing the activities. Fig. 3 also demonstrated that DTA-2 exert stronger



Scheme-I: Synthesis of compounds DTA-1,2, DTA-5 and DTA-8



Scheme-II: Synthesis of compounds DTA-3,4, DTA-6,7 and DTA-9

protective effect than DTA-4, therefore, DTA-2 was chosen for further evaluation.

Given that DTA-2 was composed of DTM and ACS48, we further compared its protective effect with SAB, DTM, ACS48 and the mixture of DTM and ACS48. As shown in Fig. 4, cell viability was significantly decreased after *t*-BHP treatment, while DTA-2 treatment concentration-dependently improved the cell viability. At a concentration of 100 μM, the

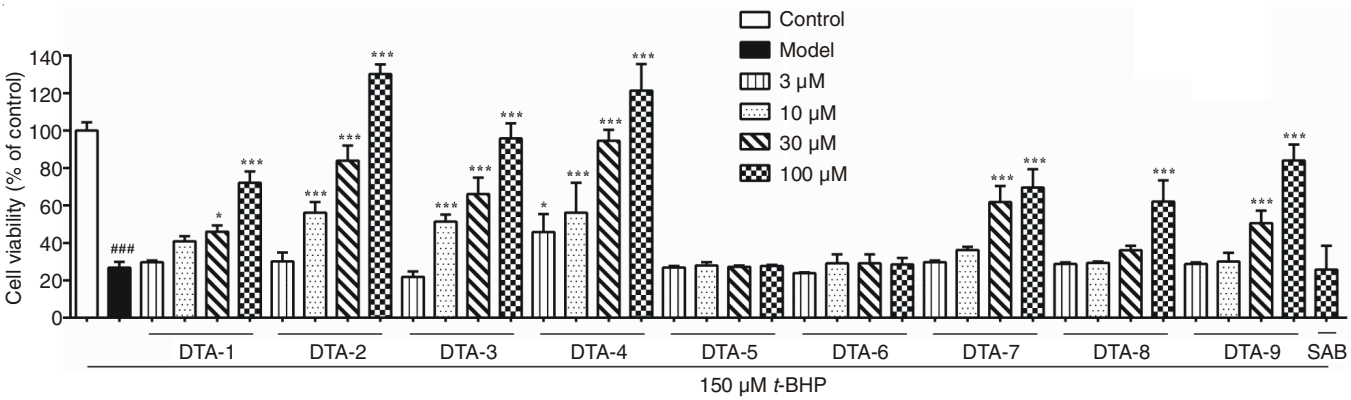


Fig. 3. Effects on *t*-BHP injured cells

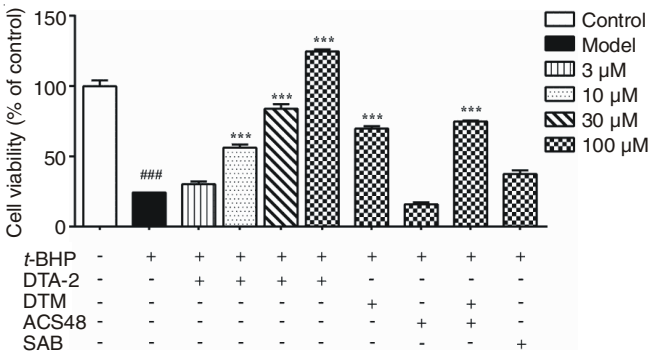


Fig. 4. Protective effect on *t*-BHP injured H9c2 cells

protective effect of DTA-2 was much stronger than that of SAB. Moreover, the protective effect of DTA-2 was stronger than those of DTM, ACS48 and the mixture of the two compounds. The results demonstrated that the improved protective effect of DTA-2 was a synergistic effect of DTM and ACS48.

Free radicals play pivotal roles in the pathogenesis of myocardial ischemia/reperfusion injury and a number of antioxidants have displayed therapeutic effects [27]. DTA-2 was the conjugate of DSS, TMP and H_2S donors, which were found to be effective in scavenging free radicals [28,29], thus, DTA-2 was more likely to have the similar activities. We then further investigated its free radical scavenging effect. The levels of free radicals stimulated by *t*-BHP were reflected by the intensity of DCF fluorescence and the results were shown in Fig. 5. DTA-2 was found to be effective in scavenging free radicals in a concentration-dependent manner. At a concentration of 100 μM, the radical scavenging effect of DTA-2 was more potent than those of DTM, ACS48 and the mixture of DTM with ACS48, suggested an additive effect was obtained.

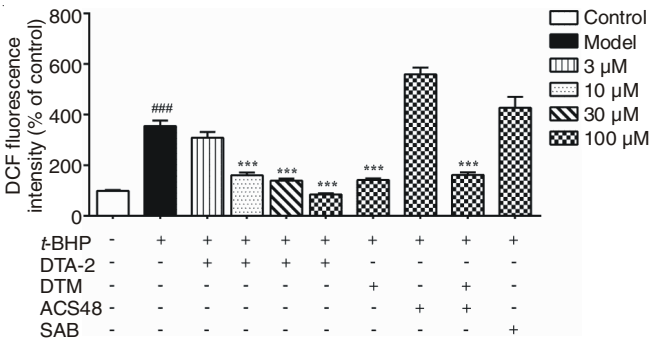


Fig. 5. Free radical scavenging effect in cultured cells

Mitochondria are the main source of free radicals and their damage will result in release of apoptosis factors that will cause myocardial cell apoptosis which is the main pathological mechanism of myocardial ischemia/reperfusion injury [30]. Mitochondria structure damage usually accompany with mitochondrial membrane potential (MMP) decrease[31]. Thus, to evaluate the protective effect of DTA-2 on mitochondria, we further studied the protective effect of DTA-2 on MMP. Results in Fig. 6 showed that *t*-BHP treatment induced a loss of MMP, while DTA-2 treatment effectively inhibited the MMP loss. DTM and the mixture of DTM and ACS48 showed similar activities to that of DTA-2 at a concentration of 100 μM.

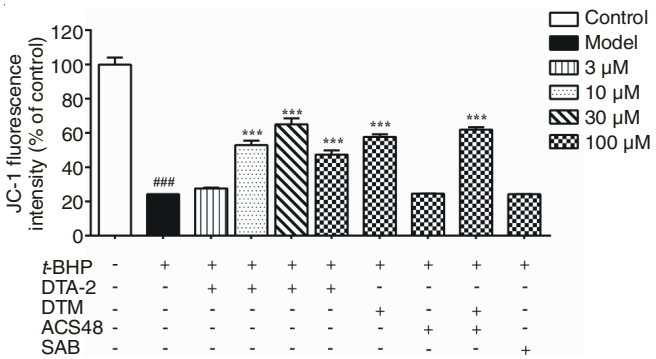


Fig. 6. Effect on mitochondrial membrane potential (Δψm)

Hydrogen sulfide donor ACS48, however, showed no effects in these tests above. While compound DTA-2 showed better protective effects than the mixture of ACS48 and DTM. All these results above indicated that combing hydrogen sulfide donor ACS48 with DTM produced synergic effects and DTA-2 may act through a different pathway that needs further investigation.

Conclusion

In summary, we have synthesized several novel conjugates combining DSS, TMP and H_2S donors and evaluated their biological activities *in vitro*. Their protective effects on cellular oxidative injury were observed. Among the new compounds, DTA-2 provided the strongest protective effect against *t*-BHP induced injury. Moreover, DTA-2 was found to be more effective in scavenging free radicals and reducing the loss of MMP than its components so, components ACS48 and DTM in DTA-2 may play synergic effects. Further studies, including the H_2S-releasing activity and the protective effect of DTA-2 in a rat model of heart ischemia/reperfusion will be reported in due course.

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