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Structure–activity studies on the protection of Trimetazidine derivatives modified with nitroxides and their precursors from myocardial ischemia–reperfusion injury

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Abstract—Trimetazidine, the known anti-anginal and anti-ischemic drug, was modified by pyrroline and tetrahydropyridine nitroxides and their hydroxylamine and sterically hindered secondary amine precursors. The synthesized new compounds proved to be better superoxide scavenger molecules compared to the parent Trimetazidine in an in vitro experiment. This reactive oxygen species (ROS) scavenging activity was further supported by ischemia/reperfusion (I/R) studies on Langendorff-perfused rat hearts pretreated with Trimetazidine and with the modified Trimetazidine derivatives before ischemia. Two of the investigated compounds, containing 2,2,5,5-tetramethyl-2,5-dihydro-1*H*-pyrrole and 4-phenyl-2,2,5,5-tetramethyl-2,5-dihydro-1*H*-pyrrole substituents on the piperazine ring, provided significant protection from the cardiac dysfunction caused by I/R. The protective effect could be attributed to the combined anti-ischemic and antioxidant effects.

1. Introduction

Trimetazidine (**1**) is widely used in heart therapy as an anti-ischemic agent devoid of hemodynamic effects.[1] This compound inhibits the 3-ketoacyl-coenzyme A thiolase activity and leads to a shift in energy substrate preference: instead of fatty acid oxidation, glucose oxidation is favored.[2]

Trimetazidine reduces intracellular acidosis and electrolyte abnormalities by optimizing the oxygen demand of the mitochondria and also inhibits ATP depletion.[3,4] It was reported that Trimetazidine inhibits the formation of reactive oxygen species (ROS) and also protects cells from ROS-induced injuries.[5] It has been shown that Trimetazidine protects the heart from ischemia-induced arrhythmias, reduces infarct size, and preserves the effects of ischemic and pharmacological preconditioning.[6] These anti-ischemic effects of Trimetazidine could be useful for the treatment of ischemic heart disease[7] and it was found to be effective in the treatment of stable angina, alone, or when combined with conventional anti-anginal agents,[8] although Trimetazidine was reported to cause reversible parkinsonism.[9]

Trimetazidine's relatively simple structure (1-[2,3,4-trimethoxy benzyl] piperazine) inspired pharmaceutical chemists to introduce substituents on the secondary amine of the piperazine ring. The most successful derivative is probably lomerizine 1-[bis(4-fluorophenyl)methyl]-4-[(2,3,4-trimethoxyphenyl)methyl] piperazine, which was first introduced as an anti-migraine drug.[10] Other modifications include acylation of the secondary nitrogen of piperazine with either 2,3-dihydro-1,4-benzodioxin or 1,4-benzodioxin derivatives which were then used as lipid peroxidation inhibitors.[11] Trimetazidine derivatives modified by a tocopherol or hydroquinone moiety were found to be active against lipid peroxidation and albumin oxidation.[12] Flavonoid derivatives, coupled with 2,3,4-trimethoxybenzyl piperazines, were found to be effective modulators of multi-drug resistance.[13] Two experimental Trimetazidine derivatives S-15176 (2,6-di-*tert*-butyl-4-{3-[4-(2,3,4-trimethoxy-benzyl)-piperazin-1-yl] propylsulfanyl}phenol) and S-16950 (6-(4-benzyl-piperazin-1-ylmethyl)-2,3-dimethoxyphenol)

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were capable of inhibiting the loss of ATP synthesis observed in isolated mitochondria exposed to cyclosporin A (CSA), an immunosuppressive drug.[14] These two compounds are good candidates for use in combination with CSA because they counteract the deleterious effects of CSA on mitochondrial membranes, without decreasing the immunosuppressive effect of CSA. Considering the advantages of the aforementioned modifications of Trimetazidine, it was a real challenge to synthesize new derivatives of Trimetazidine which preserved the original activity, but exhibited better antioxidant activity than the parent drug. The introduction of paramagnetic five- and six-membered nitroxides (1-oxyl-2,2,5,5-tetramethyl-2,5-dihydro-1*H*-pyrrole or 1-oxyl-2,2,6,6-tetramethyl-1,2,3,6-tetrahydro-pyridine rings) and their reduced forms (amines or hydroxylamines) might accomplish this. The advantage of these modifications in cardiac drugs is the protection from the cell damage caused by ROS during ischemia.[15,16] Alkylation of the mexiletine amino group with a 2,2,5,5-teramethyl-pyrroline-3-yl methyl group yielded a new compound that provided more protection from ischemia–reperfusion-induced contractile dysfunction in isolated hearts compared to the parent drug, mexiletine.[17] Modification of the diethylaminoethyl side chain of amiodarone with this same 2,2,5,5-tetramethylpyrrolin-3-ylmethyl group resulted in a compound which has enhanced activity and less toxicity, as its inhibitory effect on mitochondrial permeability is similar to that of amiodarone, but without biphasic characteristics.[18]

Our laboratory has reported very recently that the substitution of the *N*-phenyl group of ebselen with α,α′-tetramethyl pyrroline, pyrrolidine, or tetrahydropyridine nitroxides and their diamagnetic precursors exhibited better glutathione peroxidase-like (GP*x*) activity than the ebselen itself.[19]

Our current studies have been focused on alkylation or acylation of the piperazine secondary amine group to create a new series of paramagnetic and diamagnetic Trimetazidine derivatives. We also investigated their superoxide-scavenging activity and their protection of hearts from ischemia/reperfusion-induced contractile dysfunction.

2. Results and discussion

2.1. Chemistry

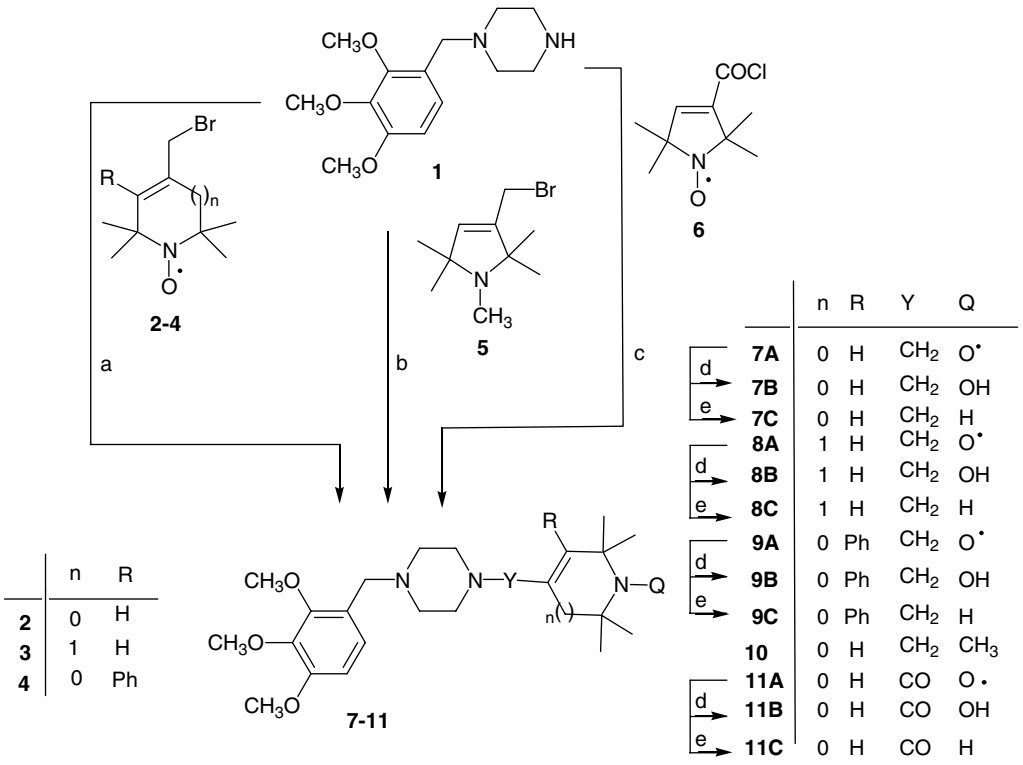
The alkylation of the piperazine NH of Trimetazidine (**1**) with a five-membered paramagnetic allylic bromide **2**,[20] a six-membered allylic bromide **3**,[21] and a five-membered allylic bromide with a 4-phenyl substituent **4**,[22] in $CHCl_3$ in the presence of K_2CO_3 gave nitroxides **7A**, **8A**, and **9A**, respectively. Treatment of compound **1** with an *N*-methylpyrroline allylic bromide **5** in dioxane in the presence of K_2CO_3 produced compound **10**. The acylated derivative **11A** was obtained by treatment of **1** with paramagnetic acyl chloride[23] (**6**) in CH_2Cl_2 in the presence of Et_3N. To increase the water solubility of these compounds, salts of the diamagnetic reduced forms were prepared. The treatment of nitroxides **7**–**9A**, **11A** with HCl saturated EtOH[15] gave the corresponding hydroxylamine/HCl salts **7B**, **8B**, **9B**, and **11B**, respectively. The reduction of nitroxides with Fe/AcOH[24] yielded the secondary amines **7**–**9C** and **11C**, which were also used in the biological investigations as HCl salt (Scheme 1).

2.2. Scavenging of superoxide radical

To compare the efficiency of **1** and novel Trimetazidine derivatives in scavenging superoxide, EPR spectroscopy was used to study the formation of DEPMPO–OOH adducts (Fig. 1). DEPMPO will scavenge superoxide and form DEPMPO–OOH adduct which can be measured by EPR. The formation of superoxide radicals was generated by xanthine/xanthine oxidase. The addition of SOD inhibited the EPR signal almost 95%, indicating that the formed adduct was from superoxide radicals. The adduct formed when Trimetazidine or one of its derivatives scavenges the superoxide radicals is not detectable by EPR. When Trimetazidine or one of its derivatives is combined with a reaction mixture containing DEPMPO, they compete to scavenge the superoxide radicals. When less DEPMPO–OOH is formed, the EPR signal is inhibited. The signal was not significantly inhibited by the Trimetazidine (**1**), however, the compounds being investigated: **7B**, **7C**, **8B**, **8C**, **9C**, **11B**, and **11C** exhibited superoxide-scavenging activity because it was shown that less DEMPO–OOH adduct was formed. Compounds **7B**, **8B**, **9C**, and **11C** were found to have the greatest effect, for example, both the hydroxylamines and the sterically hindered amines were effective in scavenging superoxides. It was reported that sterically hindered amines eliminate the superoxide.[25,26] In the present case, we observed that compound **1**, containing a secondary NH in the piperazine ring, does not have significant superoxide-scavenging activity, while the sterically hindered secondary amines **7C**, **8C**, **9C**, and **11C** are better superoxide scavengers. The reaction of nitroxide or hydroxylamine with superoxide is well documented[27,28] (Fig. 2). Nitroxides catalyze the formation of O_2 and H_2O_2 from superoxide. All of the derivatives exhibited ROS scavenging activity as shown by the reduction in the amount of DEPMPO–OOH adduct formed. Based on these results of the in vitro experiments, we concluded that further ex vivo studies for both the hydroxylamines, 'B form' and the sterically hindered amines, 'C form' would be useful.

2.3. Langendorff-heart experiments

Isolated rat hearts were first perfused with compounds **1**, **7B**, **7C**, **8B**, **8C**, **9C**, **10**, **11B**, or **11C** (50 μM) for 1 min before ischemia and then subjected to global ischemia followed by reperfusion. Coronary flow (CF), left ventricular developed pressure (LVDP), heart rate (HR), and rate pressure product (RPP, defined as a product of heart rate and LVDP) were measured prior to the start of global ischemia and during reperfusion. The functional recovery of the CF, LVDP, and RPP data obtained during reperfusion was expressed as a percentage of pre-ischemic baseline values (Fig. 3).



Scheme 1. Structures and synthesis of Trimetazidine and its derivatives. Reagents and conditions: (a) compounds **2**–**4** (1.1 equiv) K_2CO_3, $CHCl_3$, reflux, 3 h, 49–72%; (b) compound **5** (1.0 equiv) K_2CO_3, dioxane, and 18-crown-6 (cat.), reflux, 3 h, 39%; (c) compound **6**, Et_3N (1.1 equiv), CH_2Cl_2 0 °C to room temperature, 1 h, 63%; (d) EtOH (saturated with HCl gas) reflux, 30 min then Et_2O, 52–73%; (e) AcOH, Fe powder (10 equiv) 70 °C, 1 h, then water, decant, K_2CO_3 extracted by $CHCl_3$, 33–62%.

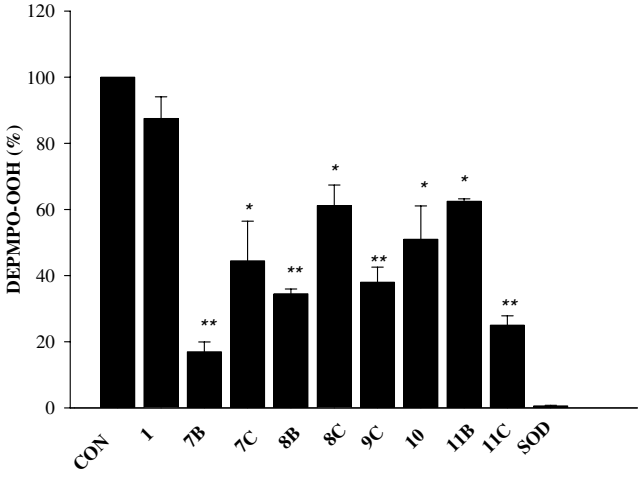


Figure 1. Scavenging of superoxide by **1** and its derivatives. Superoxide radicals were generated by xanthine (0.5 mM) and XO (0.02 U/ml). The addition of SOD almost completely inhibited the EPR signal, indicating the formed adduct originated from superoxide radicals. The reaction mixture containing DTPA (0.1 mM), DEPMPO (10 mM) in air-saturated PBS, (pH 7.4) in the presence of 1 mM of **1** or one of its derivatives was measured after 15 min of incubation using EPR spectroscopy. Data represent means ± SD ($n = 4$), $^*p < 0.01$ versus control (CON), $^{**}p < 0.001$ versus control (CON).

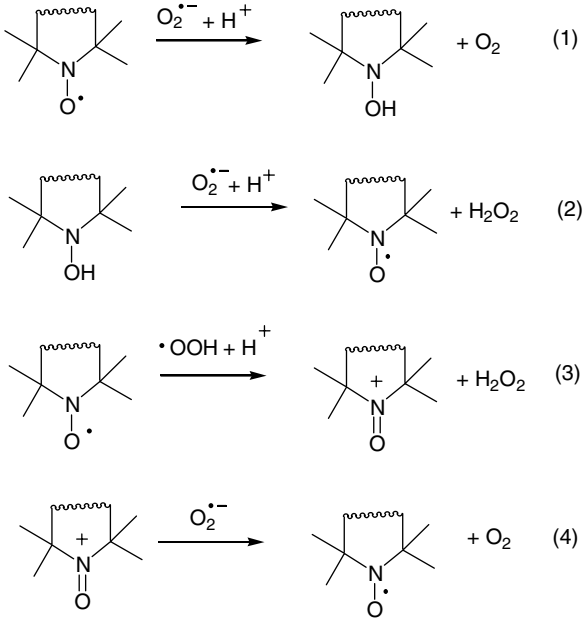


Figure 2. Possible reactions of a superoxide anion radical with a nitroxide or a hydroxylamine.

The pre-ischemic baseline values were as follows: CF: 17 ± 4 ml/min; LVDP: 124 ± 18 mmHg; and HR, 274 ± 24 bpm. There was an insignificant drop, initially, in the LVDP and the heart rate in hearts pretreated with the five-membered pyrroline nitroxide-containing compounds **7C** and **9C** (data not shown). However, no significant differences were observed in the recovery of coronary flow when we compared the control, Trimetazidine **1**, and its derivatives. Trimetazidine **1** and

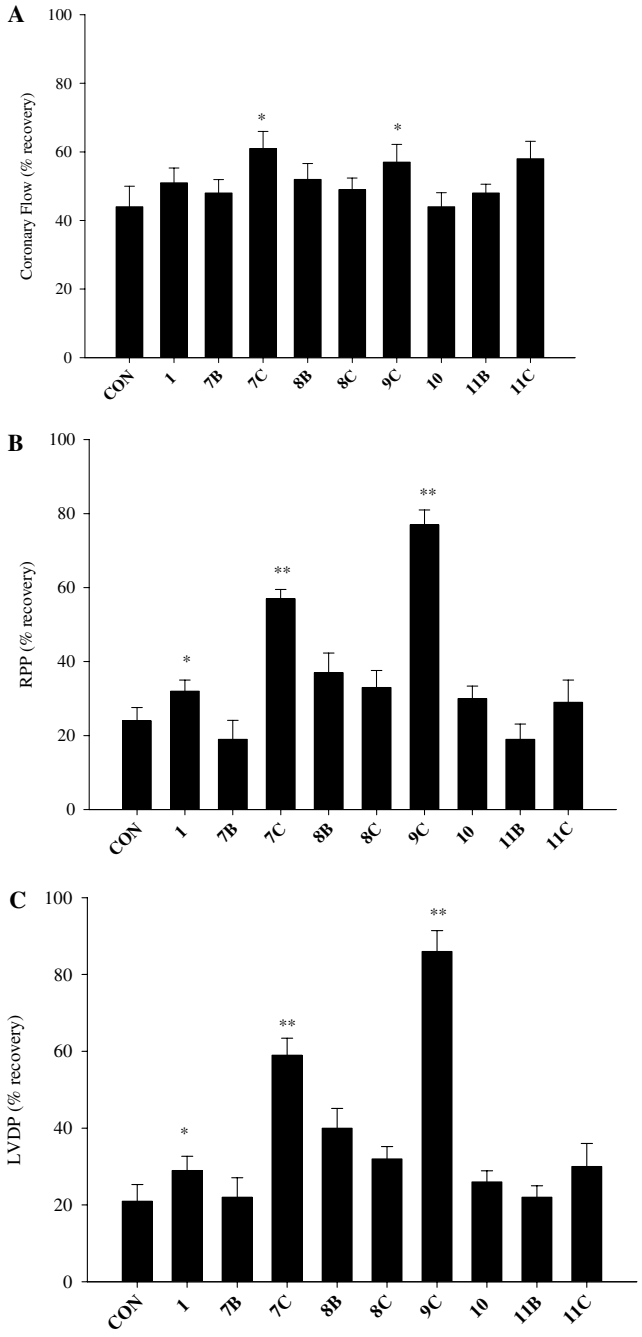


Figure 3. Effect of Trimetazidine (**1**) and its derivatives on the recovery of (A) coronary flow (CF); (B) rate pressure product (RPP) and (C) left ventricular developed pressure (LVDP). Rat hearts were pretreated with 50 μM **1** or its derivatives for 1 min before ischemia. Values obtained from six independent experiments are expressed as means ± SD. *$p < 0.05$ versus control (CON), **$p < 0.001$ versus control (CON).

compounds **7B**, **8B**, **8C**, **10**, **11B**, and **11C** had influence on the RPP and LVDP (Figs. 3b and c), but their effect was not significant compared to control. The most effective compound (**9C**) was obtained when a phenyl substituent, that is, 4-phenyl-2,2,5,5-tetramethyl-2,5-dihydro-1*H*-pyrrole, was introduced on the pyrroline ring. This resulted in a significant enhancement of contractile function as evidenced by the greatly increased percent of recovery in the RPP and LVDP. It is probably due to the fact that compound **9C** contains both a lipophilic aromatic group and a hydrophilic amino group with the ability to scavenge reactive oxygen species (ROS), including superoxides, which are formed during ischemia/reperfusion (I/R) in both the lipid membrane and the hydrophilic cytosolic areas.

3. Conclusions

This work supports our previous observation that the modification of a cardiac drug with nitroxides or their reduced forms (hydroxylamines or sterically hindered secondary amines) will be more beneficial since these modified drugs inhibit the damages caused by ROS during ischemia formed in statu nascendi. All of the examined derivatives scavenged superoxide in an in vitro assay, however the studies in the Langendorff-perfused heart experiments demonstrated that two compounds, both with a 2,2,5,5-tetramethyl-2,5-dihydro-1*H*-pyrrol-3ylmethyl substituent on the piperazine nitrogen, show promise for further investigations.[29]

4. Materials and methods

Melting points were determined with a Boetius micro-melting point apparatus and are uncorrected. Elemental analyses (C, H, N, and S) were performed on a Fisons EA 1110 CHNS elemental analyzer. Mass spectra were recorded on a Thermoquest Automass Multi and VG TRIO-2 instrument in the EI or thermospray (TSP) mode. ^{1}H NMR spectra were recorded with a Varian $^{\text{UNITY}}$*INOVA* 400 WB spectrometer. Chemical shifts are referenced to Me_4Si. Measurements were run at a 298 K probe temperature in D_2O solution. EPR spectra were taken on a Miniscope MS 200 in 10^{-4} M $CHCl_3$ solution, and all monoradicals gave a triplet line a_N = 14.7–15.1 G. Flash column chromatography was performed on a Merck Kieselgel 60 (0.040–0.063 mm). Qualitative TLC was carried out on commercially prepared plates (20 × 20 × 0.02 cm) coated with Merck Kieselgel GF_{254}.

Compounds **1**,[30] **2**,[18] **3**,[21] **4**,[22] **5**,[31] and **6**[24] were prepared according to published procedures. All reagents and solvents were purchased from Aldrich.

4.1. General procedure for alkylation of Trimetazidine (7A, 8A, and 9A)

A mixture of an allylic bromide **2**, **3** or **4** (11.0 mmol), K_2CO_3 (1.51 g, 11.0 mmol), and **1** (2.66 g, 10.0 mmol) in $CHCl_3$ (20 mL) was stirred and refluxed until the starting materials were consumed (~2–3 h). After cooling, the inorganic salts were filtered out, the organic phase was washed with brine (15 mL), separated, dried with $MgSO_4$, filtered, evaporated, and then the residue was purified by flash column chromatography ($CHCl_3$/Et_2O) to give the title compounds as yellow or red oils 49–72%.

4.1.1. 2,2,5,5-Tetramethyl-3-[4-(2,3,4-trimethoxybenzyl)-piperazin-1-ylmethyl]-2,5-dihydro-1*H*-pyrrol-1-yloxyl radical (7A, HO-2921). Yellow oil, 2.34 g, (56%). MS (EI) *m*/*z* (%): 418 (M^+, 3), 388 (10), 207 (48), 181 (100). Anal. Calcd for $C_{23}H_{36}N_3O_4$: C, 66.00; H, 8.67; N, 10.04. Found: C, 65.88; H, 8.50; N, 10.01.

4.1.2. 2,2,6,6-Tetramethyl-4-[4-(2,3,4-trimethoxybenzyl)-piperazin-1-ylmethyl]-1,2,3,6-terahydropyridin-1-yloxyl radical (8A, HO-2922). Red oil, 2.11 g (49%). MS (EI) *m*/*z* (%): 432 (M^+, 2), 417 (8), 221 (19), 181 (100). Anal. Calcd for $C_{24}H_{38}N_3O_4$: C, 66.64; H, 8.85; N, 9.71. Found: C, 66.59; H, 8.77; N, 9.62.

4.1.3. 4-Phenyl-2,2,5,5-tetramethyl-3-[4-(2,3,4-trimethoxybenzyl)-piperazin-1-ylmethyl]-2,5-dihydro-1*H*-pyrrol-1-yloxyl radical (9A, HO-3629). Yellow oil, 3.56 g (72%). MS (EI) *m*/*z* (%): 494 (M^+, 3), 464 (6), 283 (55), 181 (100). Anal. Calcd for $C_{29}H_{40}N_3O_4$: C, 70.42; H, 8.15; N, 8.49. Found: C, 70.39; H, 8.02; N, 8.31.

4.2. Synthesis of 1,2,2,5,5-pentamethyl-3-[4-(2,3,4-trimethoxybenzyl)-piperazin-1-yl methyl]-2,5-dihydro-1*H*-pyrrole (10, HO-3615)

A mixture of compound **1** (1.33 g, 5.0 mmol), compound **5** (1.16 g, 5.0 mmol), K_2CO_3 (690 mg, 5.0 mmol), 18-crown-6 (20 mg) in dioxane (15 mL) was stirred and refluxed for 4 h. After cooling, the inorganic salts were filtered off, the dioxane was evaporated, the residue was partitioned between water (10 mL) and $CHCl_3$ (20 mL), then the organic phase was separated, dried with $MgSO_4$, filtered, and evaporated. The residue was purified by column chromatography ($CHCl_3$/Et_2O) to give the title compound as a colorless oil 811 mg, 39%. This base was dissolved in EtOH saturated with HCl, and after evaporation of the solvent, the residue was crystallized from Et_2O to yield a white solid, mp 173–175 °C. MS (EI) *m*/*z* (%): 431 (M^+, 1), 402 (21), 181 (71), 138 (100). ^{1}H NMR (D_2O), 400 MHz: δ = 7.16 (d, J = 8.8 Hz, 1H, Ar*H*), 6.88 (d, J = 8.8 Hz, 1H, Ar*H*), 6.05 (s, 1H, =C*H*), 4.34 (s, 2H, ArCH_2N), 3.88 (s, 3H, OCH_3), 3.83 (s, 3H, OCH_3), 3.81 (s, 3H, OCH_3), 3.72 (s, 2H, CH=CH–CH_2), 3.49 (br s, 4H, piperazine CH_2), 3.32 (br s, 4H, piperazine CH_2), 2.71 (s, 3H, NCH_3) 1.48 (s, 3H, CH_3), 1.46 (s, 3H, CH_3), 1.41 (s, 3H, CH_3), 1.38 (s, 3H, CH_3). Anal. Calcd for $C_{24}H_{42}Cl_3N_3O_3$: C, 54.70; H, 8.03; N, 7.97. Found: C, 54.56; H, 7.92; N, 7.90.

4.3. (1-Oxyl-2,2,5,5-tetramethyl-2,5-dihydro-1*H*-pyrrol-3-yl)-[4-(2,3,4-trimethoxy-benzyl)-piperazin-1-yl]methanone radical (11A, HO-3594)

A fifteen mL solution of Et_3N (555 mg, 5.5 mmol) and acyl-chloride **6** (1.11 g, 5.5 mmol) in CH_2Cl_2 was added dropwise to a stirred solution of compound **1** (1.33 g, 5.0 mmol), in CH_2Cl_2 at 0 °C. After stirring the mixture at room temperature for 1 h, the yellow solution was washed with brine (10 mL), the organic phase was separated, dried with $MgSO_4$, filtered, and evaporated. The residue was purified by column chromatography (hexane/EtOAc) to give the title compound as a thick, yellow oil 1.36 g, (63%). MS (EI) *m*/*z* (%): 432 (M^+, 1), 402 (4), 219 (14), 181 (100). Anal. Calcd for $C_{23}H_{34}N_3O_5$: C, 63.87; H, 7.92; N, 9.71. Found: C, 63.80; H, 7.85; N, 9.88.

4.4. General procedure for synthesis of hydroxylamines (7B, 8B, 9B, and 11B)

A solution of nitroxide **7A**, **8A**, **9A**, or **11A** (2.0 mmol) was refluxed for 30 min in EtOH (15 mL) saturated with HCl. After cooling, the solvent was evaporated off, and then the residue was crystallized from Et_2O or acetone to give the title compounds as white crystals 52–73%.

4.4.1. 1-Hydroxy-2,2,5,5-tetramethyl-3-[4-(2,3,4-trimethoxybenzyl)-piperazin-1-ylmethyl]-2,5-dihydro-1*H*-pyrrole 3HCl salt (7B, HO-2921/OH/3HCl). White solid, 773 mg (73%), mp 206–208 °C. ^{1}H NMR (D_2O), 400 MHz: δ = 7.16 (d, J = 8.8 Hz, 1H, Ar*H*), 6.89 (d, J = 8.8 Hz, 1H, Ar*H*), 5.82 (s, 1H, =C*H*), 4.29 (s, 2H, ArCH_2N), 3.88 (s, 3H, OCH_3), 3.83 (s, 3H, OCH_3), 3.81 (s, 3H, OCH_3), 3.49 (s, 2H, CH=CH–CH_2), 3.42 (br s, 4H, piperazine CH_2), 3.16 (br s, 4H, piperazine CH_2), 1.50 (s, 6H, 2× CH_3), 1.47 (s, 6H, 2× CH_3). Anal. Calcd for $C_{23}H_{40}Cl_3N_3O_4$: C, 52.23; H, 7.62; N, 7.94. Found: C, 52.11; H, 7.59; N, 7.80.

4.4.2. 1-Hydroxy-2,2,6,6-tetramethyl-4-[4-(2,3,4-trimethoxybenzyl)-piperazin-1-ylmethyl]-1,2,3,6-terahydropyridine 3HCl salt (8B, HO-2922/OH/3HCl). White solid, 650 mg (60%), mp 192–194 °C, ^{1}H NMR (D_2O), 400 MHz: δ = 7.16 (d, J = 8.8 Hz, 1H, ArH), 6.88 (d, J = 8.8 Hz, 1H, ArH), 5.97 (s, 1H, =CH), 4.34 (s, 2H, ArCH_2N), 3.88 (s, 3H, OCH_3), 3.83 (s, 3H, OCH_3), 3.81 (s, 3H, OCH_3), 3.68 (s, 2H, CH=CH–CH_2N), 3.51 (br s, 4H, piperazine CH_2), 3.36 (br s, 4H, piperazine CH_2), 2.52 (s, 2H, CH=CH–CH_2), 1.46 (s, 6H, 2× CH_3), 1.41 (s, 3H, CH_3), 1.37 (s, 3H, CH_3). Anal. Calcd for $C_{24}H_{42}Cl_3N_3O_4$: C, 53.09; H, 7.80; N, 7.74. Found: C, 53.01; H, 7.79; N, 7.59.

4.4.3. 1-Hydroxy-4-phenyl-2,2,5,5-tetramethyl-3-[4-(2,3,4-trimethoxybenzyl)-piperazin-1-ylmethyl]-2,5-dihydro-1*H*-pyrrole 3HCl salt (9B, HO-3629/OH/3HCl). Hygroscopic white solid, 629 mg (52%). ^{1}H NMR (D_2O), 400 MHz: δ = 7.40 (s, 3H, ArH), 7.19 (d, J = 7.2 Hz, 2H, ArH), 7.05 (d, J = 8.8 Hz, 1H, ArH), 6.86 (d, J = 8.8 Hz, 1H, ArH), 4.16 (s, 2H, ArCH_2N), 3.83 (s, 6H, 2× OCH_3), 3.80 (s, 3H, OCH_3), 3.30 (s, 2H, CH=CH–CH_2N), 3.10 (br s, 4H, piperazine CH_2), 2.95 (br s, 4H, piperazine CH_2), 1.63 (s, 6H, 2× CH_3), 1.43 (s, 6H, 2× CH_3). Anal. Calcd for $C_{29}H_{44}Cl_3N_3O_4$: C, 57.57; H, 7.33; N, 6.94. Found: C, 57.55; H, 7.27; N, 6.89.

4.4.4. (1-Hydroxy-2,2,5,5-tetramethyl-2,5-dihydro-1*H*-pyrrol-3-yl)-[4-(2,3,4-trimethoxy-benzyl)-piperazin-1-yl]-methanone 2HCl salt (11B, HO3594/OH/2HCl). White solid, mp 204–206 °C, 588 mg (56%). ^{1}H NMR (D_2O), 400 MHz: δ = 7.16 (d, J = 8.8 Hz, 1H, Ar*H*), 6.89 (d, J = 8.8 Hz, 1H, Ar*H*), 6.20 (s, 1H, =C*H*), 4.30 (s, 2H, ArCH_2N), 3.88 (s, 3H, OCH_3), 3.83 (s, 3H, OCH_3), 3.82 (s, 3H, OCH_3), 3.30 (br s, 8H, piperazine

4× CH_2), 1.54 (s, 6H, 2× CH_3), 1.51 (s, 6H, 2× CH_3). Anal. Calcd for $C_{23}H_{37}Cl_2N_3O_5$: C, 54.54; H, 7.36; N, 8.30; Found: C, 54.50; H, 7.22; N, 8.28.

4.5. General procedure for synthesis of sterically hindered amines (7C, 8C, 9C, 11C)

Fe powder (1.12 g, 20 mmol) was added to a solution of nitroxide **7A**, **8A**, **9A**, or **11A** (2.0 mmol) in AcOH (10 mL) and then the mixture was warmed to 70 °C until the reaction started. The mixture was stirred at this temperature for 1 h, allowed to cool, diluted with water (15 mL), decanted, and then the decanted aqueous solution was made alkaline by adding solid K_2CO_3. The mixture was extracted with $CHCl_3$ (3× 15 mL), dried with $MgSO_4$, filtered, evaporated, and after chromatographic purification ($CHCl_3$/MeOH), the title amines **7C**, **8C**, **9C or 11C** were obtained as colorless oils 33–62%. These bases were converted to salts by dissolving in EtOH saturated with HCl gas and then evaporating the solvents.

4.5.1. 2,2,5,5-Tetramethyl-3-[4-(2,3,4-trimethoxybenzyl)-piperazin-1-ylmethyl]-2,5-dihydro-1*H*-pyrrole 3HCl salt (7C, HO-2921NH/3HCl). White solid, 600 mg (39%), mp 195–197 °C. MS: $[M + H]^+$: 404. ^{1}H NMR (D_2O), 400 MHz: δ = 7.16 (d, J = 8.8 Hz, 1H, ArH), 6.89 (d, J = 8.8 Hz, 1H, ArH), 5.83 (s, 1H, =CH), 4.31 (s, 2H, ArCH_2N), 3.88 (s, 3H, OCH_3), 3.83 (s, 3H, OCH_3), 3.81 (s, 3H, OCH_3), 3.49 (s, 2H, CH=CH–CH_2), 3.42 (br s, 4H, piperazine CH_2), 3.16 (br s, 4H, piperazine CH_2), 1.51 (s, 6H, 2× CH_3), 1.49 (s, 6H, 2× CH_3). Anal. Calcd for $C_{23}H_{40}Cl_3N_3O_3$: C, 53.86; H, 7.86; N, 8.19. Found: C, 53.83; H, 7.82; N, 8.05.

4.5.2. 2,2,6,6-Tetramethyl-4-[4-(2,3,4-trimethoxybenzyl)-piperazin-1-ylmethyl]-1,2,3,6-tetrahydropyridine 3HCl salt (8C, HO-2943/3HCl). White solid, 348 mg (33%), mp 199–201 °C. MS: TSP: $[M+H]^+$: 418. ^{1}H NMR (D_2O), 400 MHz: δ = 7.16 (d, J = 8.8 Hz, 1H, ArH), 6.88 (d, J = 8.8 Hz, 1H, ArH), 5.96 (s, 1H, =CH), 4.29 (s, 2H, ArCH_2N), 3.88 (s, 3H, OCH_3), 3.83 (s, 3H, OCH_3), 3.81 (s, 3H, OCH_3), 3.68 (s, 2H, CH=CH–CH_2N), 3.50 (br s, 4H, piperazine CH_2), 3.34 (br s, 4H, piperazine CH_2), 2.29 (s, 2H, CH=CH–CH_2), 1.44 (s, 6H, 2× CH_3), 1.38 (s, 6H, 2× CH_3). Analy. Calcld for $C_{24}H_{42}Cl_3N_3O_3$: C, 54.70; H, 8.03; N, 7.97. Found: C, 54.66; H, 7.90; N, 7.81.

4.5.3. 4-Phenyl-2,2,5,5-tetramethyl-3-[4-(2,3,4-trimethoxybenzyl)-piperazin-1-ylmethyl]-2,5-dihydro-1*H*-pyrrole 3HCl salt (9C, HO-3630/3HCl). White solid, 730 mg (62%), mp 178–180 °C. MS (EI) m/z (%): 479 (M^+, 4), 298 (41), 212 (53), 181 (100). ^{1}H NMR (D_2O), 400 MHz: δ = 7.39 (s, 3H, ArH), 7.19 (d, J = 7.2 Hz, 2H, ArH), 7.07 (d, J = 8.8 Hz, 1H, ArH), 6.87 (d, J = 8.8 Hz, 1H, ArH), 4.16 (s, 2H, ArCH_2N), 3.83 (s, 6H, 2× OCH_3), 3.80 (s, 3H, OCH_3), 3.30 (s, 2H, CH=CH–CH_2N), 3.12 (br s, 4H, piperazine CH_2), 2.96 (br s, 4H, piperazine CH_2), 1.65 (s, 6H, 2× CH_3), 1.45 (s, 6H, 2× CH_3). Anal. Calcd for $C_{29}H_{44}Cl_3N_3O_3$: C, 59.13; H, 7.53; N, 7.13. Found: C, 59.08; H, 7.38; N, 7.04.

4.5.4. (2,2,5,5-Tetramethyl-2,5-dihydro-1*H*-pyrrol-3-yl)-[4-(2,3,4- trimethoxybenzyl)-piperazin-1-yl]methanone 2HCl salt (11C, HO-3595/2HCl). White solid, 539 mg (55%), mp 217–219 °C. MS: TSP: $[M + H]^+$: 418. ^{1}H NMR (D_2O), 400 MHz: δ = 7.16 (d, J = 8.8 Hz, 1H, ArH), 6.89 (d, J = 8.8 Hz, 1H, ArH), 6.07 (s, 1H, =CH), 4.29 (s, 2H, ArCH_2N), 3.88 (s, 3H, OCH_3), 3.88 (s, 3H, OCH_3), 3.83 (s, 3H, OCH_3), 3.81 (s, 3H, OCH_3), 3.30 (br s, 8H, piperazine 4× CH_2), 1.59 (s, 6H, 2× CH_3), 1.55 (s, 6H, 2× CH_3). Analy. Calcd for $C_{23}H_{37}Cl_2N_3O_4$: C, 56.32; H, 7.60; N, 8.57. Found: C, 56.28; H, 7.54; N, 8.46.

4.6. Isolated rat heart preparation

Sprague–Dawley rats (body weight 300–350 g) were first administered 60 mg/kg sodium pentobarbital (anesthesia) and 500 IU/kg heparin (anti-coagulant), intraperitoneally. After a midline sternotomy, the hearts were rapidly excised and then perfused retrogradely at a constant perfusion pressure of 80 mmHg with a modified Krebs solution containing NaCl (120 mM), $NaHCO_3$ (25 mM), $MgSO_4$ (1.2 mM), KH_2PO_4 (1.2 mM), $CaCl_2$ (1.2 mM), and glucose (11 mM). The perfusate buffer was saturated with a 95% O_2 and 5% CO_2 gas mixture at 37 °C. A latex balloon was inserted in the left ventricle via the left atrium and inflated with 0.4 ml of distilled water, this will produce an end diastolic pressure of 8–12 mmHg. The contractile and hemodynamic functions of the heart were continuously monitored with a computer-based data acquisition system (PC PowerLab with Chart 5 software, ADI Instruments, Colorado Springs, CO). The following data were measured: coronary flow (CF), left ventricular developed pressure (LVDP), and heart rate (HR). The rate pressure product (RPP) was calculated as LVDP × HR. The coronary flow rate was measured using a flowmeter with an in-line probe (Transonic Systems Inc., Ithaca, NY).

4.7. I/R experimental protocol

Isolated rat hearts were perfused for 15 min to stabilize the functions. Then the hearts were randomly divided into: (i) control group, received no treatment; or (ii) pre-treated with **1**, **7B**, **7C**, **8B**, **8C**, **9C**, **10**, **11B**, or **11C**. The drugs, as water soluble hydrochloride salts (50 μM), were dissolved in Krebs buffer and then infused through the side arm at the rate of 1 ml/min for one min before ischemia (pH 7.4). Finally, the hearts were subjected to 30-min of ischemia, followed by 45-min of reperfusion.

4.8. Superoxide scavenging using in vitro study

The superoxide-scavenging properties of **1**, **7B**, **7C**, **8B**, **8C**, **9C**, **10**, **11B**, or **11C** were evaluated by using EPR spectroscopy. Xanthine (0.5 mM) and xanthine oxidase (0.02 U/ml), in PBS, at a pH of 7.4 were used to generate superoxide radicals. The reaction mixture contained 0.1 mM DTPA, 10 mM DEPMPO, and PBS (pH 7.4), in presence or absence of 1 mM of either **1**, **7B**, **7C**, **8B**, **8C**, **9C**, **10**, **11B**, or **11C**. In combination, these mixtures result in varying amounts of superoxide radicals

which can be detected as DEPMPO–OOH adducts using X-band EPR spectroscopy. From this, the amount (%) of adduct in the mixture can be determined.

4.9. Data analysis

The statistical significance of the results of the assays was evaluated by using ANOVA and Student's *t*-test. All of the values are expressed as means ± SD. A p value <0.05 was considered significant.

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