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Ligustrazine derivatives. Part 3: Design, synthesis and evaluation of novel acylpiperazinyl derivatives as potential cerebrocardiac vascular agents ☆

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ABSTRACT

A series of novel acylpiperazinyl Ligustrazine derivatives was designed, synthesized, and their protective effects on damaged ECV-304 cells and antiplatelet aggregation activities were evaluated. The results showed that compound **E33** displayed most potential protective effects on the ECV-304 cells damaged by hydrogen peroxide, and compound **E1** was found to be the most active antiplatelet aggregation agent. Structure–activity relationships were briefly discussed.

1. Introduction

Substituted pyrazine derivatives play a very important role in pharmaceutical research for the treatment of cardiovascular disorders. Ligustrazine (Lig; tetramethylpyrazine, TMP; see Fig. 1) is a cardiovascular drug containing a pyrazine ring, which is widely used in China for the treatment of coronary atherosclerotic cardiovascular disease and ischemic cerebrocardiac vascular disease.[2]

Ligustrazine has been reported to inhibit the platelet aggregation,[3] to cause negative chronotropic and inotropic responses on isolated atria,[4] to inhibit vasoconstriction in isolated vascular strips,[5] and to act as a vasodilator, a free radical scavenger, antithrombosis and anti-hypertention agent.[2] More recently, it has been found to be more effective in protection against vascular endothelial cell injury.[6]

However, pharmacokinetics studies found that Ligustrazine presented low bioavailability and to be metabolized fast in vivo with short half-life of $T_{1/2}$ = 2.89 h, so accumulated toxicity often appeared in the patients for keeping an effective plasma concentration by the frequent administration.[7] Therefore, it is necessary to develop new generation of the cerebrocardiac vascular drugs from molecular modification of Ligustrazine.

Structure–activity relationship studies indicated that pyrazine ring in the molecule of Ligustrazine might largely be the determinant of its pharmacodynamics, while the substituted groups might primarily govern its pharmacokinetics and toxicity.[8]

According to the principles of hypridization and bioisosteric replacement in medicinal chemistry, some drug-like groups and pharmacophores can be introduced to the methyl position of Ligustrazine, for acquiring the pharmacologically additive or synergetic effects to improve pharmacokinetic properties.

Calcium channel antagonists, such as Cinnarizine and Flunarizine (see Fig. 1) are very important cerebrocardiac vascular drugs currently used in the clinic. A piperazine moiety as a linker is the common characters existed in their molecular structures, which is considered as the functional group for keeping the drugs' potential.[9] Based on the structures of the calcium antagonists above, Ligustrazine were modified by combination with a piperazine and some pharmacophores or drug-like groups, such as acetylsalicyloyl, nicotinoyl, cinnamoyl, furoyl and salicyloyl to form the new integrated structural pattern (see Fig. 1). Some substituted benzoyl groups were also introduced in order to further expand our exploration and better understand the structure–activity relationships of Ligustrazine derivatives.

2. Chemistry

In the synthesis, 2-hydroxymethyl-3,5,6-trimethylpyrazine (**B**) was prepared by the Boekelheide reaction starting from Ligustrazine trihydrate (**A**), but it was used one-pot reaction according to our previous publication with 64% of the total yield (mp 88–89 °C).[10] The important intermediate of 2-chloromethyl-3,5,6-

☆ See Ref. 1.

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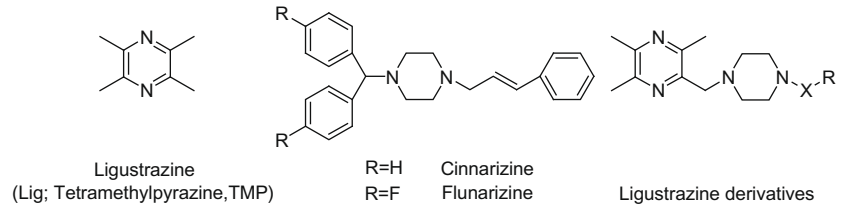
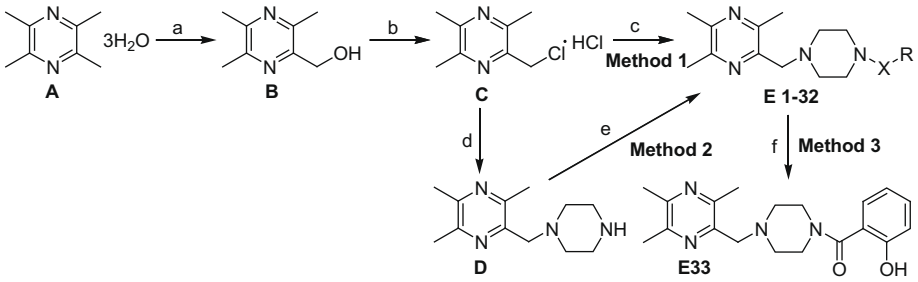


Figure 1. Structures of Ligustrazine, Cinnarizine, Flunarizine and newly synthesized acylpiperazinyl Ligustrazine derivatives.



Scheme 1. Reagents and conditions: (a) Boekelheide reaction; (b) $SOCl_2$/anhydrous CH_2Cl_2; (c) various mono N-substituted acylpiperazines, toluene, NaI, Et_3N/reflux for 10 h; (d) piperazine; (e) RCOCl, anhydrous CH_2Cl_2/reflux for 10 h; (f) RX = acetylsalicyloyl, $NH_3 \cdot H_2O$.

trimethylpyrazine hydrochloride (**C**) was synthesized by the chlorination of **B** with $SOCl_2$ in anhydrous CH_2Cl_2 (see Scheme 1).[11]

Acylpiperazinyl Ligustrazine derivatives (**E1–33**) were synthesized by the following three methods. In method 1,2-chloromethyl-3,5,6-trimethylpyrazine hydrochloride (**C**) was directly reacted with the mono-substituted *N*-acylpiperazines, which were prepared in our lab, to afford acylpiperazinyl Ligustrazine derivatives (**E10**–**22**, **24**, **26**–**30**, see Method 1 of Scheme 1).[12,13] In Method 2, 2-chloromethyl-3,5,6-trimethylpyrazine hydrochloride (**C**) alkylated with anhydrous piperazine in chloroform to produce the intermediate 2-(1-piperazinmethyl)-3,5,6-trimethylpyrazine (**D**), which was reacted with various acyl chloride to give the corresponding acylpiperazinyl Ligustrazine derivatives (**E1**–**9**, **23**, **25**, **31**, **32**, see Method 2 of Scheme 1).[13] In method 3, **E33** was obtained by hydrolysis of **E1** in ammonium hydroxide medium (see Method 3 of Scheme 1). The chemical structures of these compounds were confirmed by IR, ^1H NMR and ESI-MS.

3. Biological evaluation and discussion

Endothelial cells play a critical physiological role in maintaining normal vessel and organ function. Much evidence showed that vascular endothelial cells damage that causes the alteration of endothelial permeability barrier and vascular tone is a major promoter of atherogenesis, thrombosis and consequently cardiovascular events. Oxidative stress is a cardiovascular risk factor and contributes significantly to endothelial injury during atherogenesis. Therefore, the protection of endothelial cells against damage caused by oxidative stress is a very important therapeutic strategy.[14]

The newly synthesized Ligustrazine derivatives were assayed for the protective effects on the human umbilical vascular endothelial cells (ECV-304 cells) damaged by hydrogen peroxide. The results (Table 1) showed that Ligustrazine and most of its derivatives presented protective effects on the damaged ECV-304 cells and some of the Ligustrazine derivatives were more active (with lower EC_{50} values) than Ligustrazine (EC_{50} value at 154.35 μM).

The derivatives **E3**, **E10**, **E11**, **E20**, **E30** and **E33** exhibited high potency with EC_{50} values below 100 μM, among which **E33** containing salicyloyl group was the most active one with the EC_{50} value at 6.13 μM. The excellent potency of **E33** might be caused by the hydroxyl group at phenyl moiety, as the acetylated counterpart **E1** showed less potency with the EC50 value at 159.24 μM.

Introduction of *iso*-nicotinoyl group (**E3**) favored the protective effects on ECV-304 cells, however, replacement of this group by nicotinoyl group produced **E2** with tenfold less potency.

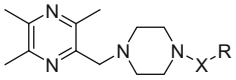
Furoyl group, galloyl group, and 2-(4-chlorophenoxy)-2-methyl-propanoyl group are confirmed as pharmacophores of cerebrocardiac vascular profiles. Introduction of furoyl group (**E10**) and galloyl group (**E11**) produced protective effect on ECV-304 cells. However, introduction of 2-(4-chlorophenoxy)-2-methyl-propanoyl pharmacophore (**E4**) did not show protective effect with EC_{50} value not determined.

Among compounds **E12**, **E24** and **E26**, the activity order was **E26** > **E24** > **E12**, which might reflect the importance of carbon numbers between the phenyl group and carbonyl group. Two carbons between the phenyl group and carbonyl group seem most favorable. In addition, the compounds containing cinnamoyl or sulfonyl group exhibited less potency as compared with Ligustrazine. And the benzoyl series compounds did not present satisfactory potency with the exception of **E20**.

Ligustrazine has been reported to inhibit the platelet aggregation,[3] so these Ligustrazine derivatives were further tested for antiplatelet aggregation induced by adenosine diphosphate (ADP).[15] The preliminary antiplatelet aggregation results obtained from the experiments showed that Ligustrazine derivatives bearing pharmacophores or drug-like groups, such as **E1** (acetylsalicyloyl, 3.02 ± 3.78%), **E2** (nicotinoyl, 5.08 ± 1.45%), **E3** (*iso*-nicotinoyl, 6.07 ± 6.48%), **E6** ((E)-3,4-dimethoxylcinnamoyl, 6.13 ± 7.23%), **E8** (acetylferuloyl, 5.84 ± 4.21%) exhibited the high potent activities in antiplatelet aggregation at 200 μM, and with lower values than that of Ligustrazine (7.86 ± 4.17%). Compound **E1** (acetylsalicyloyl, 3.02%) was found to be the most active antiplatelet aggregation agent (Table 1).

Deacetylation of **E1** produced the compound **E33** (salicyloyl, 16.71 ± 2.13%), which caused a decreased antiplatelet aggregation activity. Most of the substituted benzoyl derivatives showed higher antiplatelet aggregation activities than the non-substituted ben-

Table 1
The structures, EC_{50} for protecting damaged ECV-304 cells and platelet aggregation rate (*A*%) of Liqustrazine derivatives **E1**–**33**



No.	X	RX-	Method	EC_{50} (μM)	*A*% (200 μM)[a]
E1	CO	Acetylsalicyloyl	2	159.24	3.02 ± 3.78
E2	CO	Nicotinoyl	2	220.02	5.08 ± 1.45
E3	CO	*iso*-Nicotinoyl	2	29.73	6.07 ± 6.48
E4	CO	2-(4-Chlorophenoxy)-2-methyl-propanoyl	2	ND	13.68 ± 5.17
E5	CO	(*E*)-2,5-Dimethoxylcinnamoyl	2	376.28	9.78 ± 3.51
E6	CO	(*E*)-3,4-Dimethoxylcinnamoyl	2	282.47	6.13 ± 7.23
E7	CO	(*E*)-2,3-Dimethoxylcinnamoyl	2	340.48	8.78 ± 1.97
E8	CO	Acetylferuloyl	2	ND	5.84 ± 4.21
E9	CO	(*E*)-3-Nitrocinnamoyl	2	421.50	13.07 ± 5.54
E10	CO	Furoyl	1	37.57	21.76 ± 5.87
E11	CO	3,4,5-Trimethoxylbenzoyl	1	88.73	14.26 ± 4.43
E12	CO	Benzoyl	1	240.57	28.46 ± 8.57
E13	CO	2-Methoxylbenzoyl	1	248.56	24.27 ± 2.76
E14	CO	4-Methoxylbenzoyl	1	210.47	26.06 ± 5.58
E15	CO	3,4-Dimethoxylbenzoyl	1	169.95	29.06 ± 4.67
E16	CO	2-Chlorobenzoyl	1	161.14	27.24 ± 4.97
E17	CO	3-Chlorobenzoyl	1	121.46	24.74 ± 6.43
E18	CO	4-Chlorobenzoyl	1	208.03	25.62 ± 2.09
E19	CO	2,4-Dichlorobenzoyl	1	248.56	16.08 ± 6.83
E20	CO	2-Iodobenzoyl	1	67.80	25.45 ± 5.54
E21	CO	4-Iodobenzoyl	1	125.33	25.17 ± 8.79
E22	CO	4-Nitrobenzoyl	1	461.88	28.64 ± 7.24
E23	CO	3,5-Dinitrobenzoyl	2	178.81	15.67 ± 2.98
E24	CO	Phenylacetyl	1	188.61	25.59 ± 1.85
E25	CO	4-Nitrophenylacetyl	2	376.93	18.24 ± 3.16
E26	CO	β-Phenylpropionoyl	1	150.00	13.29 ± 4.95
E27	SO_2	Benzenesulfonyl	1	480.30	24.49 ± 7.04
E28	SO_2	4-Methylbenzenesulfonyl	1	ND	25.13 ± 5.83
E29	SO_2	Methanesulfonyl	1	162.22	33.51 ± 3.95
E30	CO	Exthoxycarbonyl	1	76.59	15.25 ± 6.89
E31	CO	Chloroacetyl	2	147.52	24.89 ± 4.29
E32	CO	Phenylethyldione	2	304.37	26.11 ± 5.07
E33	CO	Salicyloyl	3	6.13	16.71 ± 2.13
Lig				154.35	7.86 ± 4.17

ND: not determined.
[a] The *A*% value for blank is 0.73 ± 2.93, and for ADP is 39.77 ± 3.69.

zoyl derivatives (**E12**), but with some exceptions of 3,4-dimethoxy, 4-nitryl substituents. Among the compounds **E12**, **E24** and **E26**, there were increasing antiplatelet aggregation activities with the increasing length of side chain at the phenyl moiety, which might reflect the importance of alkyl chain length of the aralkyl carboxylic acid. All features described above should be considered in the design of novel Ligustrazine derivatives.

4. Conclusion

In conclusion, a series of novel acylpiperazinyl Ligustrazine derivatives was designed and synthesized. Activity assay identified compound **E1** as the most active antiplatelet aggregation agent, and compound **E33** with most potent protective effect on the damaged ECV-304 cells. Structure–activity relationships were briefly discussed. Further bioassay of these compounds on cerebrocardiac vascular activity on animal models is underway.

5. Experimental

5.1. Synthetic methods and spectroscopic details

Infrared spectra were measured using a nicolet nexus 470 FT-IR spectrometer using smear KBr crystal or KBr plate. ^{1}H NMR spectra were recorded on a Bruker Avance (600 MHz) spectrometer; *J* values are in hertz. ^{13}C NMR spectra were also recorded on the Bruker Avance spectrometer. Mass spectra were recorded on an electro-spray ionization mass spectrometer as the value *m*/*z*. Thin-layer chromatography (TLC) was performed on E. Merck Silica Gel 60-F-254 plates. Flash chromatography was performed using 300 mesh silica gel. The yields were calculated by the last step reaction.

5.1.1. (3,5,6-Trimethylpyrazin-2-yl)methanol (B)

2,3,5,6-Tetramethylpyrazine trihydrate (**A**) (30.40 g, 160 mmol) was heated with 30% hydrogen peroxide (18 ml, 160 mmol) in glacial acetic acid (40 ml) for 4 h at 70 °C. Further 30% hydrogen peroxide (18 ml, 160 mmol) was then added and the heating was continued for 4 h. The solution was made alkaline with 50% sodium hydroxide and extracted with chloroform. The combined extracts were dried and evaporated in vacuo; tetramethylpyrazine mono-N-oxide was obtained. To this, acetic anhydride (15.1 ml, 160 mmol) was added and the mixture was refluxed for 3 h, checking for product formation via TLC. The excess of acetic anhydride was evaporated and (3,5,6-trimethylpyrazin-2-yl)methyl acetate was obtained, which was directly saponified with 20% NaOH (155 ml), and extracted with chloroform. The combined extracts were dried and the solvent removed. The residual oil was recrystallized from *n*-hexane; (3,5,6-trimethylpyrazin-2-yl)methanol (**B**) was obtained as yellow needles (15.50 g, 64%); mp 88–89 °C (lit.[10] 88–89 °C).

5.1.2. 2-Chloromethyl-3,5,6-trimethylpyrazine hydrochloride (C)

Thionyl chloride (7.41 ml, 102 mmol) was added dropwise to (3,5,6-trimethylpyrazin-2-yl)methanol (**B**) (15.50 g, 102 mmol) in anhydrous CH_2Cl_2 (300 ml) at 0 °C. The mixture was allowed to stand for 2.5 h, checking for product formation via TLC. The solvent was evaporated in vacuo and the crude product 2-chloromethyl-3,5,6-trimethylpyrazine hydrochloride (**C**) was obtained as a yellow solid (21.11 g, 100%); mp 102–105 °C. Compound **C** was basified and then purified to give 2-chloromethyl-3,5,6-trimethylpyrazine as oil for spectral confirmation. IR (KBr, cm^{-1}): 2993 (CH), 2952 (CH), 2923 (CH), 2856 (CH), 1548 (C=N); ^1H NMR ($CDCl_3$, δ): 4.68 (s, 2H, CH_2), 2.63 (s, 3H, CH_3), 2.54 (s, 3H, CH_3), 2.52 (s, 3H, CH_3); ^{13}C NMR ($CDCl_3$, δ): 151.6 (C=N), 149.3 (C=N), 149.0 (C=N), 146.3 (C=N), 44.8 (CH_2), 21.6, 21.4 and 20.5 (3 × CH_3); ESI-MS: 171 (M+1).

5.1.3. 2,3,5-Trimethyl-6-(piperazin-1-ylmethyl)pyrazine (D)

2-Chloromethyl-3,5,6-trimethylpyrazine hydrochloride (**C**) (20.7 g, 100 mmol) in chloroform (100 ml) was added dropwise into anhydrous piperazine (49.88 g, 580 mmol) in chloroform (300 ml) at 0 °C. The solution was left at room temperature for 5 h, checking for product formation via TLC. The mixture solution was washed with aqueous ammonia (4 M), and the organic layers were dried. The solvent was evaporated in vacuo and the crude product was recrystallized from n-hexane to give 2,3,5-trimethyl-6-(piperazin-1-ylmethyl)pyrazine (**D**) as white crystals (11 g, 50%); mp 94 °C. IR (KBr, cm^{-1}): 3443, 3272 (NH), 2943 (CH), 1546 (C=N); ^1H NMR ($CDCl_3$, δ): 2.57–3.60 (m, 10H, CH_2), 2.49 (s, 3H, CH_3), 2.48 (s, 3H, CH_3), 2.47 (s, 3H, CH_3), 1.90 (s, 1H, NH); ^{13}C NMR ($CDCl_3$, δ): 153.6 (C=N), 150.4 (C=N), 148.7 (C=N), 147.2 (C=N), 56.6, 54.4, 54.1, 45.2 and 44.4 (5 × CH_2), 21.4, 21.0 and 20.1 (3 × CH_3); ESI-MS: 221 (M+1).

5.1.4. General procedure for the preparation of 2-(4-substituted-1-piperazinmethyl)-3,5,6-trimethylpyrazine (E10–22, E24, E26–30, Method 1 of Scheme 1)

2-Chloromethyl-3,5,6-trimethylpyrazine hydrochloride (**C**) (2.07 g, 10 mmol) and various mono N-substituted acylpiperazines (10 mmol) were dissolved in toluene (70 ml). Triethylamine (3.46 ml, 25 mmol) and NaI (catalytic quantity) were added to the solution. The mixture solution was refluxed for 10 h until the reaction was complete (monitored by TLC). After cooling, the mixture was filtered and the filtrate was evaporated in vacuo. The final product was purified by flash column chromatography and recrystallization from *n*-hexane.

5.1.4.1. 2-(4-Furoyl-1-piperazinmethyl)-3,5,6-trimethylpyrazine (E10). Flash column chromatography: chloroform–acetone = 3:1; yield 51%; yellow crystal; mp 100–102 °C; IR (KBr, cm^{-1}): 3098.74 (=CH), 2948.27 (CH), 1621.05 (C=O), 1581.79 (C=C), 1292.18 (C–O), 1023.80 (C–O); ^1H NMR ($CDCl_3$, δ): 7.46 (1H, furan-2-H), 6.97 (1H, furan-4-H, J = 3.32 Hz), 6.47 (1H, furan-3-H, J_{2-3} = 1.57 Hz, J_{3-4} = 3.13 Hz), 2.50–3.78 (m, 10H, CH_2), 2.59 (s, 3H, CH_3), 2.56 (s, 3H, CH_3), 2.49 (s, 3H, CH_3); ESI-MS: 315.3 (M+1).

5.1.4.2. 2-[4-(3,4,5-Trimethoxylbenzoyl)-1-piperazinmethyl]-3,5,6-trimethylpyrazine (E11). Flash column chromatography: chloroform–acetone = 3:1; yield 44%; yellow oil; IR (KBr, cm^{-1}): 2939.22 (CH), 1636.18 (C=O), 1583.82 (C=C), 1505.51 (C=N), 1231.28 (C–O), 1127.39 (C–O); ^1H NMR ($CDCl_3$, δ): 6.62 (s, 2H, Ar-H), 3.85–3.87 (9H, OCH_3), 2.56–3.66 (m, 10H, CH_2), 2.59 (s, 3H, CH_3), 2.50 (s, 3H, CH_3), 2.48 (s, 3H, CH_3); ESI-MS: 415.3 (M+1).

5.1.4.3. 2-(4-Benzoyl-1-piperazinmethyl)-3,5,6-trimethylpyrazine (E12). Flash column chromatography: chloroform–acetone = 10:1; yield 61%; yellow crystal; mp 138–140 °C; IR (KBr, cm^{-1}): 2913.72 (CH), 1624.65 (C=O), 1604.84 (C=C), 1578.23 (C=N); ^1H NMR ($CDCl_3$, δ): 7.41 (m, 5H, Ar-H), 2.56–3.66 (m, 10H, CH_2), 2.59 (s, 3H, CH_3), 2.49 (s, 3H, CH_3), 2.48 (s, 3H, CH_3); ESI-MS: 325.5 (M+1).

5.1.4.4. 2-[4-(2-Methoxylbenzoyl)-1-piperazinmethyl]-3,5,6-trimethylpyrazine (E13). Flash column chromatography: chloroform–acetone = 8:1; yield 44%; white crystal; mp 104–105 °C; IR (KBr, cm^{-1}): 2917.00 (CH), 1622.38 (C=O), 1600.03 (C=C), 1253.43 (C–O), 1023.52 (C–O); ^1H NMR ($CDCl_3$, δ): 7.33 (t, 1H, Ar-H, J = 7.53 Hz), 7.23 (d, 1H, Ar-H, J = 7.31 Hz), 6.98 (t, 1H, Ar-H, J = 7.46 Hz), 6.90 (d, 1H, Ar-H, J = 8.32 Hz), 3.80 (3H, OCH_3), 2.40–3.70 (m, 10H, CH_2), 2.58 (s, 3H, CH_3), 2.49 (s, 3H, CH_3), 2.47 (s, 3H, CH_3); ESI-MS: 355.4 (M+1).

5.1.4.5. 2-[4-(4-Methoxylbenzoyl)-1-piperazinmethyl]-3,5,6-trimethylpyrazine (E14). Flash column chromatography: chloroform–acetone = 8:1; yield 45%; yellow crystal; mp 82–85 °C; IR (KBr, cm^{-1}): 2921.16 (CH), 1621.72 (C=O), 1573.75 (C=C), 1514.22 (C=N), 1245.92 (C–O), 1033.00 (C–O); ^1H NMR ($CDCl_3$, δ): 7.37 (d, 2H, Ar-H, J = 8.68 Hz), 6.90 (d, 2H, Ar-H, J = 8.67 Hz), 3.82 (3H, OCH_3), 3.60–3.70 (m, 10H, CH_2), 2.57 (s, 3H, CH_3), 2.49 (s, 3H, CH_3), 2.47 (s, 3H, CH_3); ESI-MS: 355.4 (M+1).

5.1.4.6. 2-[4-(3,4-Dimethoxylbenzoyl)-1-piperazinmethyl]-3,5,6-trimethylpyrazine (E15). Flash column chromatography: chloroform–acetone = 8:1; yield 43%; yellow crystal; mp 110–112 °C; IR (KBr, cm^{-1}): 2916.64 (CH), 1629.45 (C=O), 1583.80 (C=C), 1518.01 (C=N), 1266.25 (C–O), 1026.80 (C–O); ^1H NMR ($CDCl_3$, δ): 6.97 (t, 2H, Ar-H, J = 7.56 Hz), 6.85 (d, 1H, Ar-H, J = 8.12 Hz), 3.89 (6H, OCH_3), 3.60–3.70 (10H, CH_2), 2.57(s, 3H, CH_3), 2.49 (s, 3H, CH_3), 2.48 (s, 3H, CH_3); ESI-MS: 385.3 (M+1).

5.1.4.7. 2-[4-(2-Chlorobenzoyl)-1-piperazinmethyl]-3,5,6-trimethylpyrazine (E16). Flash column chromatography: chloroform–acetone = 3:1; yield 50%; yellow crystal; mp 148 °C; IR (KBr, cm^{-1}): 2953.76 (CH), 1632.68 (C=O), 1591.87 (C=C); ^1H NMR ($CDCl_3$, δ): 7.35 (m, 4H, Ar-H), 2.30–3.83 (m, 10H, CH_2), 2.58 (s, 3H, CH_3), 2.49 (s, 3H, CH_3), 2.47 (s, 3H, CH_3); ESI-MS: 359.4 (M+1).

5.1.4.8. 2-[4-(3-Chlorobenzoyl)-1-piperazinmethyl]-3,5,6-trimethylpyrazine (E17). Flash column chromatography: chloroform–acetone = 3:1; yield 41%; yellow crystal; mp 136 °C; IR (KBr, cm^{-1}): 2920.53 (CH), 1629.36 (C=O), 1594.43 (C=C), 1563.11(C=N); ^1H NMR ($CDCl_3$, δ): 7.39 (m, 2H, Ar-H), 7.33 (t, 1H, Ar-H), 7.27 (t, 1 H, Ar-H), 2.40–3.66 (m, 10H, CH_2), 2.60 (s, 3H, CH_3), 2.49 (s, 3H, CH_3), 2.48 (s, 3H, CH_3); ESI-MS: 359.4 (M+1).

5.1.4.9. 2-[4-(4-Chlorobenzoyl)-1-piperazinmethyl]-3,5,6-trimethylpyrazine (E18). Flash column chromatography: chloroform–acetone = 4:1; yield 58%; white crystal; mp 102–104 °C; IR (KBr, cm^{-1}): 2935.07 (CH), 1614.37 (C=O), 1566.14 (C=C); ^1H NMR ($CDCl_3$, δ): 7.39 (d, 2H, Ar-H, J = 8.35 Hz), 7.36 (d, 2H, Ar-H, J = 8.44 Hz), 3.40–3.77 (m, 10H, CH_2), 2.51 (s, 3H, CH_3), 2.50 (s, 3H, CH_3), 2.49 (s, 3H, CH_3); ESI-MS: 359.3 (M+1).

5.1.4.10. 2-[4-(2,4-Dichlorobenzoyl)-1-piperazinmethyl]-3,5,6-trimethylpyrazine (E19). Flash column chromatography: chloroform–acetone = 2:1; yield 51%; yellow crystal; mp 142–144 °C; IR (KBr, cm^{-1}): 2916.04 (CH), 1631.66 (C=O), 1587.77 (C=C), 1553.47 (C=N); ^1H NMR ($CDCl_3$, δ): 7.43 (d, 1H, Ar-H, J = 8.00 Hz), 7.31 (t, 1H, Ar-H, J = 8.11 Hz), 7.23 (t, 1H, Ar-H, J = 5.30 Hz), 2.50–3.83 (m, 10H, CH_2), 2.58 (s, 3H, CH_3), 2.49 (s, 3H, CH_3), 2.48 (s, 3H, CH_3); ESI-MS: 393.2 (M+1).

5.1.4.11. 2-[4-(2-Iodobenzoyl)-1-piperazinmethyl]-3,5,6-trimethylpyrazine (E20). Flash column chromatography: chloroform–acetone = 5:1; yield 42%; white crystal; mp 163–166 °C; IR (KBr, cm^{-1}): 2949.93 (CH), 1628.84 (C=O), 1583.72 (C=C); ^1H NMR ($CDCl_3$, δ): 7.82 (d, 1H, Ar-H, J = 7.92 Hz), 7.38 (t, 1H, Ar-H, J = 7.38 Hz), 7.19 (d, 1H, Ar-H, J = 6.44 Hz), 7.06 (t, 1H, Ar-H, J = 7.04 Hz), 2.30–3.81 (m, 10H, CH_2), 2.57 (s, 3H, CH_3), 2.48 (s, 3H, CH_3), 2.47 (s, 3H, CH_3); ESI-MS: 451.3 (M+1).

5.1.4.12. 2-[4-(4-Iodobenzoyl)-1-piperazinmethyl]-3,5,6-trimethylpyrazine (E21). Flash column chromatography: chloroform–acetone = 5:1; yield 52%; white crystal; mp 166–168 °C; IR (KBr, cm^{-1}): 2938.22 (CH), 1619.75 (C=O), 1586.17 (C=C); ^1H NMR ($CDCl_3$, δ): 7.75 (d, 2H, Ar-H, J = 8.26 Hz), 7.14 (d, 2H, Ar-H, J = 8.25 Hz), 2.57–3.65 (m, 10H, CH_2), 2.50 (s, 3H, CH_3), 2.48 (s, 3H, CH_3), 2.47 (s, 3H, CH_3); ESI-MS: 451.3 (M+1).

5.1.4.13. 2-[4-(4-Nitrobenzoyl)-1-piperazinmethyl]-3,5,6-trimethylpyrazine (E22). Flash column chromatography: chloroform–acetone = 8:1; yield 63%; yellow crystal; mp 151–153 °C; IR (KBr, cm^{-1}): 2919.51 (CH), 1638.20 (C=O), 1600.48 (C=C), 1521.12 (NO_2), 1350.51 (NO_2); ^1H NMR ($CDCl_3$, δ): 8.34 (d, 2H, Ar-H, J = 8.36 Hz), 7.63 (d, 2H, Ar-H, J = 8.50 Hz), 3.40–3.87 (m, 10H, CH_2), 2.65 (s, 3H, CH_3), 2.56 (s, 3H, CH_3), 2.54 (s, 3H, CH_3); ESI-MS: 370.4 (M+1).

5.1.4.14. 2-(4-Phenylacetyl-1-piperazinmethyl)-3,5,6-trimethylpyrazine (E24). Flash column chromatography: chloroform–acetone = 8:1; yield 39%; yellow crystal; mp 67–69 °C; IR (KBr, cm^{-1}): 2955.83 (CH), 1636.90 (C=O), 1602.07 (C=C); ^1H NMR ($CDCl_3$, δ): 7.30 (m, 5H, Ar-H), 3.77 (2H, Ar-CH_2), 2.30–3.69 (m, 10H, CH_2), 2.53 (s, 3H, CH_3), 2.52 (s, 3H, CH_3), 2.50 (s, 3H, CH_3); ESI-MS: 339.4 (M+1).

5.1.4.15. 2-[4-(β-Phenylpropionoyl)-1-piperazinmethyl]-3,5,6-trimethylpyrazine (E26). Flash column chromatography: chloroform–acetone = 8:1; yellow crystal; yield 47%; mp 74–76 °C; IR (KBr, cm^{-1}): 2935.95 (CH), 1630.00 (C=O); ^1H NMR ($CDCl_3$, δ): 7.27 (t, 2H, Ar-H, J = 5.15 Hz), 7.20 (d, 3H, Ar-H, J = 7.57 Hz), 2.90–3.59 (m, 8H, CH_2), 2.60 (2H, CH_2), 2.56 (s, 3H, CH_3), 2.49 (s, 3H, CH_3), 2.48 (s, 3H, CH_3), 2.44 (t, 2H, J = 4.73 Hz), 2.36 (t, 2H, J = 4.58 Hz); ESI-MS: 353.3 (M+1).

5.1.4.16. 2-(4-Benzenesulfonyl-1-piperazinmethyl)-3,5,6-trimethylpyrazine (E27). Flash column chromatography: chloroform–acetone = 1:1; yield 48%; yellow crystal; mp 118–120 °C; IR (KBr, cm^{-1}): 2919.28 (CH), 1353.05 (SO_2), 1331.33 (SO_2), 1181.05 (SO_2), 1174.51 (SO_2); ^1H NMR ($CDCl_3$, δ): 7.75 (d, 2H, Ar-H, J = 7.30 Hz), 7.59 (t, 1H, Ar-H, J = 7.31 Hz), 7.52 (t, 2H, Ar-H, J = 7.56 Hz), 3.60 (2H, CH_2), 2.60–3.05 (m, 8H, CH_2), 2.53 (s, 3H, CH_3), 2.47 (s, 3H, CH_3), 2.46 (s, 3H, CH_3); ESI-MS: 361.4 (M+1).

5.1.4.17. 2-[4-(4-Methylbenzenesulfonyl)-1-piperazinmethyl]-3,5,6-trimethylpyrazine (E28). Flash column chromatography: chloroform–acetone = 1:1; yield 46%; white crystal; mp 119–120 °C; IR (KBr, cm^{-1}): 2920.51 (CH), 1595.88 (C=C), 1662.32 (C=N), 1350.98 (SO_2), 1329.04 (SO_2), 1168.24 (SO_2), 1130.92(SO_2); ^1H NMR ($CDCl_3$, δ): 7.67 (d, 2H, Ar-H, J = 8.17 Hz), 7.37 (d, 2H, Ar-H, J = 8.03 Hz), 3.65 (2H, CH_2), 2.63–3.07 (m, 8H, CH_2), 2.63 (s, 3H, CH_3), 2.52 (s, 3H, CH_3), 2.51 (s, 3H, CH_3), 2.49 (3H, Ar-CH_3); ESI-MS: 375.3 (M+1).

5.1.4.18. 2-(4-Methanesulfonyl-1-piperazinmethyl)-3,5,6-trimethylpyrazine (E29). Flash column chromatography: chloroform–acetone = 3:1; yield 41%; white crystal; mp 116–117 °C; IR (KBr, cm^{-1}): 2947.79 (CH), 1641.98 (C=C), 1324.32 (SO_2), 1161.38 (SO_2), 1132.50 (SO_2); ^1H NMR ($CDCl_3$, δ): 3.75 (2H, CH_2), 3.31–3.32 (m, 8H, CH_2), 2.79 (3H, SO_2CH_3), 2.57 (s, 3H, CH_3), 2.54 (s, 3H, CH_3), 2.49 (s, 3H, CH_3); ESI-MS: 299.4 (M+1).

5.1.4.19. 2-(4-Exthoxycarbonyl-1-piperazinmethyl)-3,5,6-trimethylpyrazine (E30). Flash column chromatography: chloroform–acetone = 4:1; yield 58%; yellow crystal; mp 94–96 °C; IR (KBr, cm^{-1}): 2929.29 (CH), 1702.38 (C=O), 1236.10 (C–O), 1129.85 (C–O); ^1H NMR ($CDCl_3$, δ): 3.44–4.13 (m, 10H, CH_2), 2.56 (s, 3H, CH_3), 2.48 (s, 3H, CH_3), 2.47 (s, 3H, CH_3), 2.44 (2H, CH_2), 1.24 (t, 3H, CH_3, J = 7.12 Hz); ESI-MS: 293.4 (M+1).

5.1.5. General procedure for the preparation of 2-(4-substituted-1-piperazinmethyl)-3,5,6-trimethylpyrazine (E1–9, E23, E25, E31 and E32, Method 2 of Scheme 1)

To a mixture of 2,3,5-trimethyl-6-(piperazin-1-ylmethyl)pyrazine (**D**) (2.2 g, 10 mmol) and Na_2CO_3 (3.18 g, 30 mmol) in anhydrous CH_2Cl_2 (100 ml), was added dropwise various acyl chloride (10 mmol) in anhydrous CH_2Cl_2 (100 ml) at room temperature. The mixture was refluxed for 10 h (checked by TLC), and the solvent was evaporated in vacuo. The final product was purified by flash column chromatography and recrystallization from *n*-hexane. **E1–4**, **E11–15**, **E29**, **E31**, E37 and **E38** are obtained.

5.1.5.1. 2-(4-Acetylsalicyloyl-1-piperazinmethyl)-3,5,6-trimethylpyrazine (E1). Flash column chromatography: chloroform–acetone = 3:1; yield 48%; yellow oil; IR (KBr, cm^{-1}): 2920.52 (CH), 1766.95 (C=O), 1640.86 (C=O), 1606.93 (C=N), 1195.56 (C–O); ^1H NMR ($CDCl_3$, δ): 7.29 (m, 1H, Ar-H), 7.19 (dd, 1H, Ar-H), 7.14 (t, 1H, Ar-H, J = 7.42 Hz), 7.05 (d, 1H, Ar-H, J = 8.12 Hz), 3.18–3.66 (m, 10H, CH_2), 2.47 (s, 3H, CH_3), 2.37 (s, 3H, CH_3), 2.34 (s, 3H, CH_3), 2.16 (t, 3H, $COCH_3$, J = 4.98 Hz); ESI-MS: 383.3 (M+1).

5.1.5.2. 2-(4-Nicotinoyl-1-piperazinmethyl)-3,5,6-trimethylpyrazine (E2). Flash column chromatography: chloroform–acetone = 1:1; yield 44%; white crystal; mp 120–121 °C; IR (KBr, cm^{-1}): 2916.66 (CH), 1630.39 (C=O), 1590.11 (C=C), 1567.99 (C=N); ^1H NMR ($CDCl_3$, δ): 8.65 (m, 2H, Ar-H), 7.73 (m, 1H, Ar-H), 7.34 (m, 1H, Ar-H), 3.42–3.77 (m, 10H, CH_2), 2.57 (s, 3H, CH_3), 2.49 (s, 3H, CH_3), 2.47 (s, 3H, CH_3); ESI-MS: 326.5 (M+1).

5.1.5.3. 2-(4-*iso*-Nicotinoyl-1-piperazinmethyl)-3,5,6-trimethylpyrazine (E3). Flash column chromatography: chloroform–acetone = 1:1; yield 42%; yellow crystal; mp 156 °C; IR (KBr, cm^{-1}): 2939.60 (CH), 1629.98 (C=O), 1599.69 (C=C), 1551.20 (C=N); ^1H NMR ($CDCl_3$, δ): 8.68 (q, 2H, Ar-H, J = 1.58 Hz), 7.27 (q, 2H, Ar-H, J = 1.58 Hz), 2.43–3.78 (m, 10H, CH_2), 2.57 (s, 3H, CH_3), 2.49 (s, 3H, CH_3), 2.47 (s, 3H, CH_3); ESI-MS: 326.5 (M+1).

5.1.5.4. 2-[4-[2-(4-Chlorophenoxy)-2-methyl-]propanoyl-1-piperazinmethyl]-3,5,6-trimethylpyrazine (E4). Flash column chromatography: chloroform–acetone = 5:1; yield 49%; white crystal; mp 100–101 °C; IR (KBr, cm^{-1}): 2937.20 (CH), 1629.32 (C=O), 1596.54 (C=C), 1578.56 (C=N), 1242.16 (C–O), 1002.05 (C–O); ^1H NMR ($CDCl_3$, δ): 7.19 (m, 2H, Ar-H), 6.75 (m, 2H, Ar-H), 3.80 (s, 2H, CH_2), 3.63 (s, 2H, CH_2), 3.50 (s, 2H, CH_2), 2.51 (s, 3H, CH_3), 2.48 (s, 3H, CH_3), 2.46 (s, 3H, CH_3), 2.41 (s, 2H, CH_2), 2.19 (s, 2H, CH_2), 1.62 (s, 6H, $C(CH_3)_2$); ESI-MS: 417.6 (M+1).

5.1.5.5. 2-[4-[(*E*)-2,5-Dimethoxylcinnamoyl]-1-piperazinmethyl]-3,5,6-trimethylpyrazine (E5). Flash column chromatography: chloroform–acetone = 1:1; yield 56%; yellow oil; IR (KBr, cm^{-1}): 2919.80 (CH), 1645.01 (C=O), 1604.46 (C=C), 1496.96 (C=N), 1222.62 (C–O), 1044.67 (C–O), 985.00 (=CH); ^1H NMR ($CDCl_3$, δ): 7.86 (d, 1H, =CH, J = 15.57 Hz), 7.02 (d, 1H, Ar-H, J = 2.74 Hz), 6.95 (d, 1H, =CH, J = 15.60 Hz), 6.85 (m, 2H, Ar-H), 3.83 (s, 3H,

OCH_3), 3.81 (s, 3H, OCH_3), 3.60–3.80 (m, 10H, CH_2), 2.54 (s, 3H, CH_3), 2.49 (s, 3H, CH_3), 2.48 (s, 3H, CH_3); ESI-MS: 411.6 (M+1).

5.1.5.6. 2-[4-[(*E*)-3,4-Dimethoxylcinnamoyl]-1-piperazinmethyl]-3,5,6-trimethylpyrazine (E6). Flash column chromatography: chloroform–acetone = 2:1; yield 52%; yellow oil; IR (KBr, cm^{-1}): 2935.64 (CH), 1645.14 (C=O), 1597.84 (C=C), 1513.44 (C=N), 1262.19 (C–O), 1024.92 (C–O), 999.80 (=CH); ^1H NMR ($CDCl_3$, δ): 7.59 (d, 1H, =CH, J = 15.34 Hz), 7.09 (dd, 1H, Ar-H, J = 8.29 Hz), 7.02 (d, 1H, Ar-H, J = 1.57 Hz), 6.84 (d, 1H, Ar-H, J = 8.29 Hz), 6.70 (d, 1H, =CH, J = 15.34 Hz), 3.90 (6H, OCH_3), 2.52–3.65 (m, 10H, CH_2), 2.57 (s, 3H, CH_3), 2.53 (s, 3H, CH_3), 2.48 (s, 3H, CH_3); ESI-MS: 411.5 (M+1).

5.1.5.7. 2-[4-[(*E*)-2,3-Dimethoxylcinnamoyl]-1-piperazinmethyl]-3,5,6-trimethylpyrazine (E7). Flash column chromatography: chloroform–acetone = 2:1; yield 44%; yellow oil; IR (KBr, cm^{-1}): 2936.85 (CH), 1647.52 (C=O), 1606.19 (C=C), 1578.12 (C=N), 1268.27 (C–O), 1071.58 (C–O), 1002.77 (=CH); ^1H NMR ($CDCl_3$, δ): 7.87 (d, 1H, =CH, J = 15.64 Hz), 7.10 (d, 1H, Ar-H, J = 7.82 Hz), 7.03 (t, 1H, Ar-H, J = 8.00 Hz), 6.95 (d, 1H, =CH, J = 15.64 Hz), 6.90 (d, 1H, Ar-H, J = 8.06 Hz), 3.87 (s, 3H, OCH_3), 3.84 (3H, OCH_3), 2.54–3.70 (m, 10H, CH_2), 2.60 (s, 3H, CH_3), 2.54 (s, 3H, CH_3), 2.49 (s, 3H, CH_3); ESI-MS: 411.5 (M+1).

5.1.5.8. 2-(4-Acetylferuloyl-1-piperazinmethyl)-3,5,6-trimethylpyrazine (E8). Flash column chromatography: chloroform–acetonechloroform–acetone = 2:1; yield 42%; yellow oil; IR (KBr, cm^{-1}): 2920.41 (CH), 1765.60 (C=O), 1648.46 (C=O), 1603.67 (C=C), 1509.17 (C=N), 1260.13 (C–O), 1196.81 (C–O), 1001.28 (=CH); ^1H NMR ($CDCl_3$, δ): 7.61 (d, 1H, =CH, J = 15.38 Hz), 7.12 (d, 1H, Ar-H, J = 9.79 Hz), 7.07 (s, 1H, Ar-H), 7.03 (d, 1H, Ar-H, J = 8.14 Hz), 6.79 (d, 1H, =CH, J = 15.38 Hz), 3.86 (3H, s, OCH_3), 2.50–3.80 (m, 10H, CH_2), 2.58 (s, 3H, CH_3), 2.55 (s, 3H, CH_3), 2.50 (s, 3H, CH_3), 2.32 (s, 3H, $COCH_3$); ESI-MS: 439.6 (M+1).

5.1.5.9. 2-[4-[(*E*)-3-Nitrocinnamoyl]-1-piperazinmethyl]-3,5,6-trimethylpyrazine (E9). Flash column chromatography: chloroform–acetone = 1:2; yield 62%; yellow oil; IR (KBr, cm^{-1}): 2920.27 (CH), 1651.78 (C=O), 1610.95 (C=C), 1529.96 (NO_2), 1351.87 (NO_2), 996.53 (=CH); ^1H NMR ($CDCl_3$, δ): 8.38 (s, 1H, Ar-H), 8.19 (d, 1H, Ar-H, J = 6.88 Hz), 7.78 (d, 1H, Ar-H, J = 7.70 Hz), 7.69 (d, 1H, =CH, J = 15.46 Hz), 7.56 (t, 1H, Ar-H, J = 7.93 Hz), 6.99 (d, 1H, =CH, J = 15.46 Hz), 2.59–3.71 (m, 10H, CH_2), 2.56 (s, 3H, CH_3), 2.50 (s, 3H, CH_3), 2.49 (s, 3H, CH_3); ESI-MS: 396.4 (M+1).

5.1.5.10. 2-[4-(3,5-Dinitrobenzoyl)-1-piperazinmethyl]-3,5,6-trimethylpyrazine (E23). Flash column chromatography: chloroform–acetone = 5:1; yield 45%; yellow crystal; mp 198–199 °C; IR (KBr, cm^{-1}): 2951.71 (CH), 1639.02 (C=O), 1591.30 (C=C), 1542.23 (NO_2), 1344.54 (NO_2); ^1H NMR ($CDCl_3$, δ): 9.09 (s, 1H, Ar-H), 8.59 (d, 2H, Ar-H, J = 1.68 Hz), 2.66–3.84 (m, 10H, CH_2), 2.53 (s, 3H, CH_3), 2.50 (s, 3H, CH_3), 2.49 (s, 3H, CH_3); ESI-MS: 415.5 (M+1).

5.1.5.11. 2-[4-(4-Nitrophenylacetyl)-1-piperazinmethyl]-3,5,6-trimethylpyrazine (E25). Flash column chromatography: chloroform–acetone = 5:1; yield 41%; yellow crystal; mp 50 °C; IR (KBr, cm^{-1}): 2920.09 (CH), 1647.97 (C=O), 1604.78 (C=N), 1519.28 (NO_2), 1345.87 (NO_2); ^1H NMR ($CDCl_3$, δ): 8.20 (d, 2H, Ar-H, J = 8.52 Hz), 7.42 (d, 2H, Ar-H, J = 8.45 Hz), 3.46–3.82 (m, 10H, CH_2), 2.57 (s, 3H, CH_3), 2.50 (s, 3H, CH_3), 2.48 (s, 3H, CH_3), 2.42 (s, 2H, Ar-CH_2); ESI-MS: 384.4 (M+1).

5.1.5.12. 2-(4-Chloroacetyl-1-piperazinmethyl)-3,5,6-trimethylpyrazine (E31). Flash column chromatography: chloroform–acetone = 3:1; yield 34%; yellow crystal; mp 76–77 °C; IR (KBr, cm^{-1}): 2941.71 (CH), 1668.60 (C=O), 1654.54 (C=N); ^1H NMR ($CDCl_3$, δ): 4.06 (s, 2H, $ClCH_2$), 3.51–3.67 (m, 10H, CH_2), 2.59 (s, 3H, CH_3), 2.51 (s, 3H, CH_3), 2.49 (s, 3H, CH_3); ESI-MS: 297.5 (M+1).

5.1.5.13. 2-(4-Phenylethyldione-1-piperazinmethyl)-3,5,6-trimethylpyrazine (E32). Flash column chromatography: chloroform–acetone = 1:1; yield 47%; yellow oil; IR (KBr, cm^{-1}): 2920.22 (CH), 1680.18 (C=O), 1645.37 (C=O), 1596.29 (C=C), 1579.49 (C=N); ^1H NMR ($CDCl_3$, δ): 7.93 (d, 2H, Ar-H, J = 7.49 Hz), 7.63 (t, 1H, Ar-H, J = 7.42 Hz), 7.50 (t, 2H, Ar-H, J = 7.80 Hz), 2.40–3.80 (m, 10H, CH_2), 2.54 (s, 3H, CH_3), 2.47 (s, 3H, CH_3), 2.45 (s, 3H, CH_3); ESI-MS: 353.5 (M+1).

5.1.6. Procedure for the preparation of 2-(4-salicyloyl-1-piperazinmethyl)-3,5,6-trimethylpyrazine (E33, Method 3 of Scheme 1)

A mixture of **E1** (10 mmol) and ammonia (25–28%, 50 mmol) was stired at room temperature for 5 h and checked for product formation via TLC. When the reaction finished, the mixture was extracted by chloroform and the extraction was evaporated in vacuo. The final product was purified by flash column chromatography and **E33** was obtained. Flash column chromatography: chloroform–acetone = 1:1; yield 51%; yellow oil; IR (KBr, cm^{-1}): 3065.70 (OH), 2919.83 (CH), 1614.15 (C=O); ^1H NMR ($CDCl_3$, δ): 9.68 (s, 1H, OH), 7.26 (m, 1H, Ar-H), 7.20 (d, 1H, Ar-H, J = 7.72 Hz), 6.96 (m, 1H, Ar-H), 6.82 (t, 1H, Ar-H, J = 7.33 Hz), 2.58–3.70 (m, 10H, CH_2), 2.56 (s, 3H, CH_3), 2.48 (s, 3H, CH_3), 2.46 (s, 3H, CH_3); ESI-MS: 341.4 (M+1).

5.2. Biological evaluation

5.2.1. Protective effects on ECV-304 cells damaged by hydrogen peroxide

The ECV-304 cells were plated and grown for 24 h in cultured medium, then were switched to fresh medium in the presence of 12.5, 25, 50, 100, 200 μM Ligustrazine and its derivatives. After 0.5 h incubation, 150 μM hydrogen peroxide was added and the cells were incubated for an additional 6 h. The results were expressed as the values of absorbance at 570 nm. The date are means ± SEM (n = 6). The proliferation rates of damaged cells were calculated by [OD570 (Compd)-OD570 (H_2O_2)]/[OD570 (Control)-OD570 (H_2O_2)] × 100%, which was then used to obtain EC_{50} values (see Table 1), according to the equation: $-pEC_{50} = \log C_{max} - 2\times(\sum P - 0.75 + 0.25P_{max} + 0.25P_{min})$, where C_{max} = maximum concentration, $\sum P$ = sum of proliferation rates, P_{max} = maximum value of proliferation rate and P_{min} = minimum value of proliferation rate.

5.2.2. Antiplatelet aggregation

Citric acid trisodium (3.8%, 0.3 ml), rabbit blood (2.7 ml), ADP and Ligustrazine derivatives (200 μM) were added to cuvette in sequence, another two cuvette using as blank and ADP control. The blood samples were centrifugated (270 g/5 min) and the super stratum was extracted as platelet-abundant plasma. The remnant blood samples were centrifugated again (1000g/10 min) and the super stratum was extracted as platelet penurious plasma (PPP), which was measured by aggregometer. The results were listed in Table 1 and expressed as the mean value of the platelet aggregation rate (A%, n = 6).

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