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Electronic Supplementary Information

Design, synthesis and evaluation of new ligustrazine derivatives as potential plasma-stable neuroprotective agents

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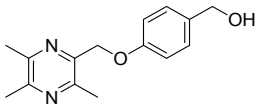
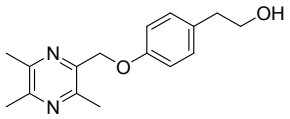
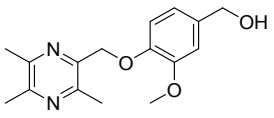
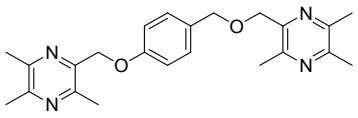
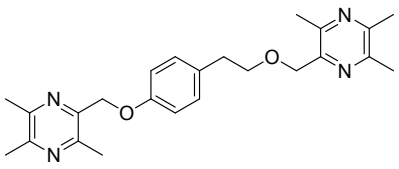
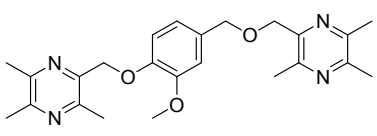
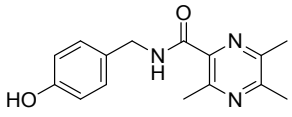
Chemistry Methods

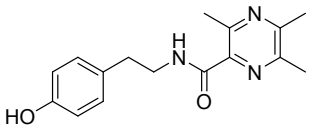
The structures and yield of all compounds were collected in Table 1

Table 1. The structures of ligustrazine-phenols derivatives.

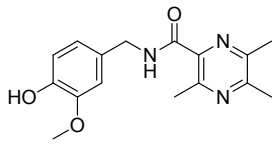
Structure	Yield	Structure	Yield
 **1a**	80.7%	 **1b**	65.7%
 **1c**	56.2%	 **2a**	64.2%
 **2b**	61.3%	 **2c**	65.0%



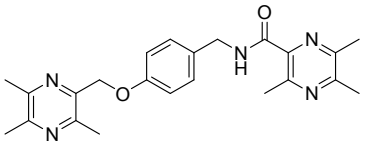
3a 93.5%



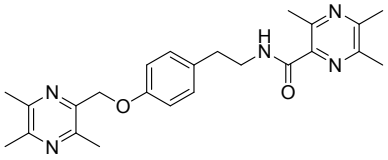
3b 88.2%



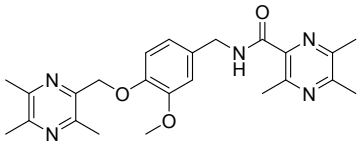
3c 82.6%



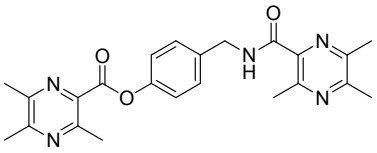
4a 86.3%



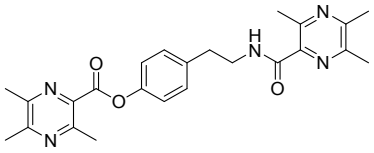
4b 77.9%



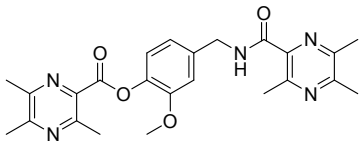
4c 85.4%



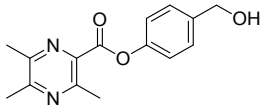
5a 72.9%



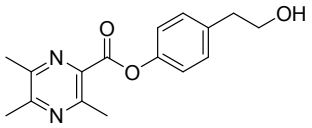
5b 75.5%



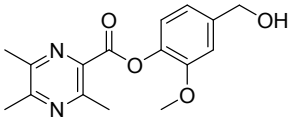
5c 70.7%



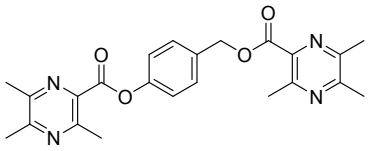
6a 46.2%



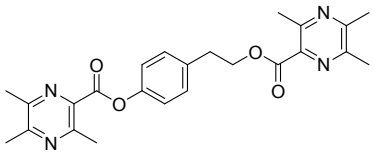
6b 40.7%



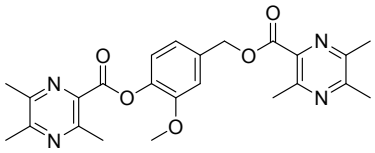
6c 45.3%



7a 53.5%



7b 49.9%



7c 39.5%

Reactions were monitored by TLC using silica gel coated aluminum sheets (Qingdao Haiyang Chemical Co., Qingdao, China) and visualized in UV light (254 nm). 1H-NMR and 13C-NMR assays

were recorded on a BRUKER AVANCE 500 NMR spectrometer (Fällanden, Switzerland) and chemical shifts are reported in d (ppm). Deuterated chloroform or deuterated dimethylsulfoxide was served as the solvent (Beijing InnoChem Science and Technology Co., Ltd., Beijing, China). HR-MS were obtained by using Q-TOF and (ESI+) with an LC Autosampler Device: Waters 2695 instrument (New York, NY, USA). Melting points (uncorrected) were measured at a rate of 5 °C/min using an X-5 micro melting point apparatus (Beijing, China). Flash chromatography was performed using 200-300 mesh silica gel. The yields were calculated based on the last step reaction. All solvents and chemicals used were analytical or high-performance liquid chromatography grade.

2-(chloromethyl)-3,5,6-trimethylpyrazine (1) [1]. Trimethylpyrazine (21.8 g, 160.0 mmol) and H_2O_2 (30%, 36 mL, 320.0 mmol) were added in AcOH (50 mL), and the mixture was stirred at 90 °C for 4 h under a nitrogen atmosphere. After the mixture cooled to room temperature, and the pH adjusted to 10.0 with sodium hydroxide solution (50 %). Extraction was performed with DCM (200 mL × 3), and the organic phase was dried with anhydrous sodium sulfate. The solvent was removed by distillation under vacuum, and the residue was dissolved in Ac_2O. Then the mixture was refluxed at 105 °C for 4 h under a nitrogen atmosphere, and adjusted the pH to 7.0 with sodium bicarbonate solution. After same post processing, the residue and NaOH (19.2 g, 480.0 mmol) was dissolved in a mixed solvent (80 mL, THF: MeOH: H_2O, 3:1:1), and stirred for 1 h. After post processing, the crude product was purified by flash chromatography. The product (13.7 g, 83.3 mmol), Thionyl chloride (6.1 mL, 83.3 mmol) were dissolved in anhydrous CH_2Cl_2 (130 mL) at 0 °C, and the mixture was stirred for 2.5 h. 5% KOH ethanol solution (80 mL) was added, and the mixture was stirred for 5 min and filtered. The filtrate was concentrated under reduced pressure. The Compound 1 (pale yellow oily liquid, 11.3 g, yield: 41.5 %, HRMS m/z: [M+H]+ calcd for $C_8H_{12}ClN_2$: 171.06890, found: 171.06873.) was purified by flash chromatography.

3,5,6-Trimethylpyrazine-2-carboxylic acid (**2**). Compound **2** was prepared according to the method described by Li et al., with minor modifications [2]. To a solution of trimethylpyrazine (5.0 g, 36.8 mmol) in water (200 mL), aqueous potassium permanganate ($KMnO_4$) solution (8.6 g $KMnO_4$: 150 mL water) was added dropwise at room temperature over about 60 min. Upon completion of the addition, the mixture was stirred at 50 °C for 12 h, and then the warm reaction mixture was filtered and washed with hot water (300 mL, 90 °C). The filtrate and washing liquor were combined, cooled to 0–5 °C, and the pH adjusted to 2.0 with concentrated hydrochloric acid. Extraction was performed with ethyl acetate (200 mL × 3), and the organic phase was dried with anhydrous sodium sulfate. The solvent was removed by distillation under vacuum, and the residue was recrystallized from acetone to produce a light yellow solid (2.39 g, yield: 47.8 %), m.p.: 162–163 °C, HRMS m/z: [M+H]+ calcd for $C_8H_{11}N_2O_2$: 167.08205, found: 167.08262.

*General procedure for the preparation of ligustrazine derivatives **1a-1c, 2a-2c** (Scheme 3)*

Compound **1** and the corresponding phenols (4-Hydroxybenzyl alcohol, 4-Hydroxyphenethyl alcohol, 4-Hydroxy-3-methoxybenzyl alcohol) were dissolved in dry DMF, then K_2CO_3 or NaH was added and the mixture was kept at 85 °C for 1.5 h under a nitrogen atmosphere. The crude product was extracted with ethyl acetate. After drying the organic layer over anhydrous Na_2SO_4 and evaporating the

solvent under vacuum, the crude products were purified by flash chromatography and recrystallization from acetone.

*(4-((3,5,6-trimethylpyrazin-2-yl) methoxy) phenyl) methanol (**1a**)*. White solid, m.p.: 92.1-92.6 °C, yield 80.7 %. 1H-NMR (500MHz, CDCl3) (ppm): 7.27 (d, j = 8.3 Hz, 2H, Ar-H), 6.97 (d, j = 8.3 Hz, 2H, Ar-H), 5.12 (s, 2H, -CH2), 4.60 (s, 2H, -CH2), 2.57 (s, 3H, -CH3), 2.51 (s, 6H, -CH3), 2.11 (s, 1H, -OH). 13C-NMR (125MHz, CDCl3) (ppm): 158.2, 151.4, 150.1, 148.8, 145.8, 133.9, 128.7, 115.0, 70.0, 64.9, 21.7, 21.5, 20.7. HRMS (ESI) m/z: $[M+H]^+$ calcd for $C_{15}H_{19}N_2O_2$: 259.14465, found: 259.14304.

*2-(4-((3,5,6-trimethylpyrazin-2-yl) methoxy) phenyl) ethanol (**1b**)*. White solid, m.p.: 91.9-92.3 °C, yield 65.7 %. 1H-NMR (500MHz, CDCl3) (ppm): 7.13 (d, j = 8.3 Hz, 2H, Ar-H), 6.95 (d, j = 8.3 Hz, 2H, Ar-H), 5.12 (s, 2H, -CH2), 3.83 (t, j = 6.4 Hz, 2H, -CH2), 2.80 (t, j = 6.4 Hz, 2H, -CH2), 2.58 (s, 3H, -CH3), 2.51 (s, 6H, -CH3), 1.61 (s, 1H, -OH). 13C-NMR (125MHz, CDCl3) (ppm): 157.4, 151.3, 150.1, 148.8, 145.9, 131.0, 130.1, 115.1, 70.1, 63.9, 38.4, 21.7, 21.4, 20.6. HRMS (ESI) m/z: $[M+H]^+$ calcd for $C_{16}H_{21}N_2O_2$: 273.16030, found: 273.15878.

*(3-methoxy-4-((3,5,6-trimethylpyrazin-2-yl) methoxy) phenyl) methanol (**1c**)*. White solid, m.p.: 112.7-113.2 °C, yield 56.2 %. 1H-NMR (500MHz, CDCl3) (ppm): 6.97 (d, j = 8.0 Hz, 1H, Ar-H), 6.92 (s, 1H, Ar-H), 6.83 (d, j = 8.0 Hz, 1H, Ar-H), 5.17 (s, 2H, -CH2), 4.60 (s, 2H, -CH2), 3.83 (s, 3H, -OCH3), 2.60 (s, 3H, -CH3), 2.49 (s, 6H, -CH3), 2.12 (s, 1H, -OH). 13C-NMR (125MHz, CDCl3) (ppm): 151.2, 150.3, 150.1, 148.6, 147.6, 145.9, 134.8, 119.3, 114.5, 110.0, 71.2, 65.3, 56.0, 21.7, 21.5, 20.7. HRMS (ESI) m/z: $[M+H]^+$ calcd for $C_{16}H_{21}N_2O_3$: 289.15520, found: 289.15387.

*2,3,5-trimethyl-6-(((4-((3,5,6-trimethylpyrazin-2-yl) methoxy) benzyl) oxy) methyl) pyrazine (**2a**)*. White solid, m.p.: 76.2-77.1 °C, yield 64.2 %. 1H-NMR (500MHz, CDCl3) (ppm): 7.27 (d, j = 8.5 Hz, 2H, Ar-H), 6.97 (d, j = 8.5 Hz, 2H, Ar-H), 5.13 (s, 2H, -CH2), 4.60 (s, 2H, -CH2), 4.51 (s, 2H, -CH2), 2.57 (s, 3H, -CH3), 2.52 (s, 3H, -CH3), 2.51 (s, 6H, -CH3), 2.49 (s, 6H, -CH3). 13C-NMR (125MHz, CDCl3) (ppm): 158.5, 151.4, 150.7, 150.1, 149.8, 148.7, 148.6, 147.2, 145.8, 130.6, 129.8, 114.9, 72.7 (-CH2), 71.6 (-CH2), 70.1 (-CH2), 21.8 (-CH3), 21.7 (-CH3), 21.5 (-CH3), 21.5 (-CH3), 20.8 (-CH3), 20.6 (-CH3). HRMS (ESI) m/z: $[M+H]^+$ calcd for $C_{23}H_{29}N_4O_2$: 393.22905, found: 393.22736.

*2,3,5-trimethyl-6-((4-(2-((3,5,6-trimethylpyrazin-2-yl) methoxy) ethyl) phenoxy) methyl) pyrazine (**2b**)*. White solid, m.p.: 83.4-84.2 °C, yield 61.3 %. 1H-NMR (500MHz, CDCl3) (ppm): 7.10 (d, j = 8.6 Hz, 2H, Ar-H), 6.90 (d, j = 8.6 Hz, 2H, Ar-H), 5.10 (s, 2H, -CH2), 4.59 (s, 2H, -CH2), 3.68 (t, j = 7.1 Hz, 2H, -CH2), 2.84 (t, j = 7.1 Hz, 2H, -CH2), 2.56 (s, 3H, -CH3), 2.50 (s, 6H, -CH3), 2.48 (s, 6H, -CH3), 2.46 (s, 3H, -CH3). 13C-NMR (125 MHz, CDCl3) (ppm): 157.2, 151.3, 150.7, 150.1, 149.9, 148.7, 148.4, 147.1, 145.9, 131.5, 129.9, 114.8, 72.7 (-CH2), 72.0 (-CH2), 70.1 (-CH2), 35.5 (-CH2), 21.8 (-CH3), 21.7 (-CH3), 21.5 (-CH3), 21.5 (-CH3), 20.8 (-CH3), 20.5 (-CH3). HRMS (ESI) m/z: $[M+H]^+$ calcd for $C_{24}H_{31}N_4O_2$: 407.24470, found: 407.24362.

*2-((2-methoxy-4-(((3,5,6-trimethylpyrazin-2-yl) methoxy) methyl) phenoxy) methyl) -3,5,6-trimethylpyrazine (**2c**)*. White solid, m.p.: 108.8-109.0 °C, yield 65.0 %. 1H-NMR (500MHz, CDCl3) (ppm): 6.98 (d, j = 8.1 Hz, 1H, Ar-H), 6.90 (s, 1H, Ar-H), 6.84 (d, j = 8.1 Hz, 1H, Ar-H), 5.18 (s, 2H, -CH2), 4.60 (s, 2H, -CH2), 4.50 (s, 2H, -CH2), 3.83 (s, 3H, -OCH3), 2.60 (s, 3H, -CH3), 2.52 (s, 3H, -CH3), 2.49 (s, 6H, -CH3), 2.48 (s, 6H, -CH3). 13C-NMR (125MHz, CDCl3) (ppm): 151.2, 150.8,

150.3, 150.0, 149.8, 148.5, 147.8, 147.1, 145.9, 131.5, 120.6, 114.3, 111.9, 72.9, 71.6, 71.2, 56.0, 21.8, 21.7, 21.5, 21.5, 20.8, 20.7. HRMS (ESI) m/z: $[M+H]^+$ calcd for $C_{24}H_{31}N_4O_3$: 423.23962, found: 423.23746.

*General procedure for the preparation of ligustrazine derivatives **3a-3c, 4a-4c, 5a-5c** (Scheme 4)*

intermediate 2 and the phenol amines (4-Hydroxybenzylamine, Tyramine, 4-Hydroxy-3-methoxybenzylamine hydrochloride) were performed using EDCI, HOBT and DIPEA in anhydrous DMF, to afford ligustrazine derivatives **3a**-**3c**. Subsequently, typical synthetic procedures for 4a-4c involved the combination of **3a**-**3c** and chloro-TMP through the formation of ether bonds under alkaline conditions. Compounds **3a**-**3c** were reacted with intermediate **2** catalyzed by EDCI and DMAP at room temperature for 12 h, respectively; we thus successfully obtained compounds **5a**-**5c**.

*N-(4-hydroxybenzyl)-3,5,6-trimethylpyrazine-2-carboxamide (**3a**)*. White solid, m.p.: 187.6-188.4 °C, yield 93.5 %. 1H-NMR (500MHz, DMSO-d6) (ppm): 9.26 (s, 1H, Ar-OH), 8.95 (t, J = 5.9 Hz, 1H), 7.11 (d, j = 8.2 Hz, 2H, Ar-H), 6.69 (d, j = 8.2 Hz, 2H, Ar-H), 4.32 (d, j = 6.2 Hz, 2H, -CH2), 2.65 (s, 3H, -CH3), 2.46 (s, 6H, -CH3). 13C-NMR (125MHz, DMSO-d6) (ppm): 164.9, 156.3, 153.4, 149.0, 147.9, 140.5, 129.6, 128.7, 115.0, 41.8, 21.9, 21.5, 20.9. HRMS (ESI) m/z: $[M+H]^+$ calcd for $C_{15}H_{18}N_3O_2$: 272.13990, found: 272.13834.

*N-(4-hydroxyphenethyl)-3,5,6-trimethylpyrazine-2-carboxamide (**3b**)*. White solid, m.p.: 177.3-177.9 °C, yield 88.2 %. 1H-NMR (500MHz, DMSO-d6) (ppm): 9.20 (s, 1H, Ar-OH), 8.61 (t, J = 5.7 Hz, 1H), 7.04 (d, j = 8.1 Hz, 2H, Ar-H), 6.69 (d, j = 8.1 Hz, 2H, Ar-H), 3.43 (dd, J = 14.2, 6.7 Hz, 2H, -CH2), 2.72 (t, j = 7.5 Hz, 2H, -CH2), 2.65 (s, 3H, -CH3), 2.49 (s, 6H, -CH3). 13C-NMR (125MHz, DMSO-d6) (ppm): 164.9, 155.7, 153.3, 148.9, 147.9, 140.4, 129.5, 129.3, 115.1, 40.6, 34.3, 21.9, 21.5, 20.9. HRMS (ESI) m/z: $[M+H]^+$ calcd for $C_{16}H_{20}N_3O_2$: 286.15555, found: 286.15378.

*N-(4-hydroxy-3-methoxybenzyl)-3,5,6-trimethylpyrazine-2-carboxamide (**3c**)*. White solid, m.p.: 151.3-152.1 °C, yield 82.6 %. 1H-NMR (500MHz, DMSO-d6) (ppm): 8.94 (s, 1H), 8.83 (s, 1H, Ar-OH), 6.91 (s, 1H, Ar-H), 6.70 (s, 2H, Ar-H), 4.34 (d, j = 5.8 Hz, 2H, -CH2), 3.72 (s, 3H, -OCH3), 2.64 (s, 3H, -CH3), 2.46 (s, 6H, -CH3). 13C-NMR (125MHz, DMSO-d6) (ppm): 165.1, 153.4, 148.9, 148.0, 147.4, 145.5, 140.7, 130.2, 119.9, 115.2, 112.0, 55.5, 42.1, 21.9, 21.6, 21.0. HRMS (ESI) m/z: [M+H]+ calcd for $C_{16}H_{20}N_3O_3$: 302.15047, found: 302.14877.

*3,5,6-trimethyl-N-(4-((3,5,6-trimethylpyrazin-2-yl)methoxy)benzyl)pyrazine-2-carboxamide (**4a**)*. White solid, m.p.: 104.8-105.7 °C, yield 86.3 %. 1H-NMR (500MHz, CDCl3) (ppm): 8.27 (s, 1H), 7.28 (d, j = 8.3 Hz, 2H, Ar-H), 6.98 (d, j = 8.3 Hz, 2H, Ar-H), 5.14 (s, 2H, -CH2), 4.56 (d, j = 5.8 Hz, 2H, -CH2), 2.93 (s, 3H, -CH3), 2.58 (s, 3H, -CH3), 2.54 (s, 3H, -CH3), 2.51 (s, 6H, -CH3), 2.48 (s, 3H, -CH3). 13C-NMR (125MHz, CDCl3) (ppm): 165.0, 158.1, 154.3, 151.6, 151.4, 150.1, 147.7, 138.9, 131.2, 129.3, 115.1, 70.1, 42.9, 23.1, 22.1, 21.8, 21.5, 21.5), 20.7. HRMS (ESI) m/z: $[M+H]^+$ calcd for $C_{23}H_{28}N_5O_2$: 406.224302, found: 406.22226.

*3,5,6-trimethyl-N-(4-((3,5,6-trimethylpyrazin-2-yl)methoxy)phenethyl)pyrazine-2-carboxamide (**4b**)*. White solid, m.p.: 148.1-148.9 °C, yield 77.9 %. 1H-NMR (500MHz, DMSO-d6) (ppm): 8.63 (s, 1H), 7.17 (d, j = 7.9 Hz, 2H, Ar-H), 6.97 (d, j = 7.9 Hz, 2H, Ar-H), 5.11 (s, 2H, -CH2), 3.46 (d, j = 6.4 Hz, 2H, -CH2), 3.34 (s, 6H, -CH3), 2.78 (t, j = 7.1 Hz, 2H, -CH2), 2.63 (s, 3H, -CH3), 2.48 (s, 3H, -

CH3), 2.45 (s, 6H, -CH3). 13C-NMR (125MHz, DMSO-d6) (ppm): 164.9, 150.8, 149.3, 148.9, 148.2, 147.8, 145.5, 131.7, 129.6, 114.6, 69.4, 40.5, 34.2, 21.8, 21.5, 21.2, 20.9, 20.1. HRMS (ESI) m/z: $[M+H]^+$ calcd for $C_{24}H_{30}N_5O_2$: 420.23995, found: 420.23706.

*N-(3-methoxy-4-((3,5,6-trimethylpyrazin-2-yl)methoxy)benzyl)-3,5,6-trimethylpyrazine-2-carboxamide (**4c**)*. White solid, m.p.: 134.3-136.2 °C, yield 85.4 %. 1H-NMR (500MHz, DMSO-d6) (ppm): 9.04 (t, J = 6.1 Hz, 1H), 7.04 (d, j = 8.2 Hz, 1H, Ar-H), 7.00 (s, 1H, Ar-H), 6.84 (d, j = 8.0 Hz, 1H, Ar-H), 5.09 (s, 2H, -CH2), 4.41 (d, j = 6.2 Hz, 2H, -CH2), 3.73 (s, 3H, -OCH3), 3.35 (s, 3H, -CH3), 2.67 (s, 3H, -CH3), 2.50 (s, 6H, -CH3), 2.45 (s, 3H, -CH3), 2.44 (s, 3H, -CH3). 13C-NMR (125MHz, DMSO-d6) (ppm): 165.1, 153.4, 150.9, 149.5, 149.2, 149.0, 148.1, 148.0, 145.5, 140.6, 132.8, 119.4, 114.1, 111.9, 70.4, 55.5, 42.0, 21.8, 21.5, 21.2, 20.9, 20.9, 20.1. HRMS (ESI) m/z: $[M+H]^+$ calcd for $C_{24}H_{30}N_5O_3$: 436.23486, found: 436.23169.

*4-((3,5,6-trimethylpyrazine-2-carboxamido)methyl)phenyl 3,5,6-trimethylpyrazine-2-carboxylate (**5a**)*. White solid, m.p.: 148.2-150.0 °C, yield 72.9 %. 1H-NMR (500MHz, DMSO-d6) (ppm): 9.22 (t, j = 6.2 Hz, 1H), 7.44 (d, j = 8.3 Hz, 2H, Ar-H), 7.25 (d, j = 8.3 Hz, 2H, Ar-H), 4.52 (d, j = 6.3 Hz, 2H, -CH2), 2.70 (s, 6H, -CH3), 2.57 (s, 3H, -CH3), 2.55 (s, 3H, -CH3), 2.51 (s, 6H, -CH3). 13C-NMR (125MHz, DMSO-d6) (ppm): 165.2, 164.1, 153.6, 149.5, 149.3, 149.2, 148.1, 140.2, 137.8, 137.5, 128.6, 121.7, 41.7, 22.2, 22.0, 21.9, 21.6, 21.0, 21.0. HRMS (ESI) m/z: $[M+H]^+$ calcd for $C_{23}H_{26}N_5O_3$: 420.20356, found: 420.20209.

*4-(2-(3,5,6-trimethylpyrazine-2-carboxamido)ethyl)phenyl 3,5,6-trimethylpyrazine-2-carboxylate (**5b**)*. White solid, m.p.: 124.7-126.2 °C, yield 75.5 %. 1H-NMR (500MHz, CDCl3) (ppm): 8.09 (s, 1H), 7.32 (d, J = 8.2 Hz, 2H, Ar-H), 7.19 (d, J = 8.2 Hz, 2H, Ar-H), 3.70 (dd, J = 13.3, 6.7 Hz, 2H, -CH2), 2.96 (t, J= 7.0 Hz, 2H, -CH2), 2.92 (s, 3H, -CH3), 2.82 (s, 3H, -CH3), 2.62 (s, 3H, -CH3), 2.61 (s, 3H, -CH3), 2.55 (s, 3H, -CH3), 2.49 (s, 3H, -CH3). 13C-NMR (125MHz, CDCl3) (ppm): 165.2, 164.7, 155.4, 154.2, 152.5, 149.7, 149.6, 139.0, 138.6, 137.1, 130.0, 122.0, 40.7, 35.5, 23.0, 22.9, 22.4, 22.1, 21.8, 21.5. HRMS (ESI) m/z: $[M+H]^+$ calcd for $C_{24}H_{28}N_5O_3$: 434.21921, found: 434.21750.

*2-methoxy-4-((3,5,6-trimethylpyrazine-2-carboxamido)methyl)phenyl 3,5,6-trimethylpyrazine-2-carboxylate (**5c**)*. White solid, m.p.: 167.5-176.9 °C, yield 70.7 %. 1H-NMR (500MHz, CDCl3) (ppm): 8.33 (s, 1H), 7.17 (d, j = 8.0 Hz, 2H, Ar-H), 7.02 (s, 1H, Ar-H), 6.99 (d, j = 8.1 Hz, 2H, Ar-H), 4.64 (d, j = 5.8 Hz, 2H, -CH2), 3.82 (s, 3H, -OCH3), 2.94 (s, 3H, -CH3), 2.82 (s, 3H, -CH3), 2.62 (s, 3H, -CH3), 2.60 (s, 3H, -CH3), 2.56 (s, 3H, -CH3), 2.51 (s, 3H, -CH3). 13C-NMR (125MHz, CDCl3) (ppm): 165.1, 164.2, 155.2, 152.5, 151.3, 149.7, 139.3, 138.8, 138.7, 137.8, 131.0, 129.0, 123.1, 120.3, 112.3, 56.1, 43.3, 23.0, 22.9, 22.4, 22.1, 21.8, 21.5. HRMS (ESI) m/z: $[M+H]^+$ calcd for $C_{24}H_{28}N_5O_4$: 450.21413, found: 450.21216.

*3.1.3. General procedure for the preparation of ligustrazine derivatives **6a-6c, 7a-7c** (Scheme 5)*

intermediate 2 and the phenols (4-Hydroxybenzyl alcohol, 4-Hydroxyphenethyl alcohol, 4-Hydroxy-3-methoxybenzyl alcohol) with different proportions (double the amount or equimolar quantities) were performed using EDCI and DMAP in anhydrous $CoCl_2$, to afford ligustrazine derivatives **6a-6c, 7a-7c**.

*4-(hydroxymethyl) phenyl 3,5,6-trimethylpyrazine-2-carboxylate (**6a**).* White solid, m.p.: 137.6–138.3 °C, yield 36.2 %. 1H-NMR (500MHz, CDCl3) (ppm): 7.43 (d, j = 8.5 Hz, 2H, Ar-H), 7.22 (d, j = 8.5 Hz, 2H, Ar-H), 4.71 (s, 2H, -CH2), 2.82 (s, 3H, -CH3), 2.62 (s, 3H, -CH3), 2.61 (s, 3H, -CH3), 2.06 (s, 1H, -OH). 13C-NMR (125MHz, CDCl3) (ppm): 164.6, 155.4, 152.5, 150.3, 149.8, 139.0, 138.5, 128.2, 122.0, 64.8, 22.9, 22.4, 21.8. HRMS (ESI) m/z: $[M+H]^+$ calcd for $C_{15}H_{17}N_2O_3$: 273.12392, found: 273.12369.

*4-(2-hydroxyethyl) phenyl 3,5,6-trimethylpyrazine-2-carboxylate (**6b**).* White solid, m.p.: 153.1-153.7 °C, yield 20.7 %. 1H-NMR (500MHz, CDCl3) (ppm): 7.29 (d, j = 7.6 Hz, 2H, Ar-H), 7.19 (d, j = 7.6 Hz, 2H, Ar-H), 3.87 (t, j = 6.4 Hz, 2H, -CH2), 2.89 (t, j = 6.4 Hz, 2H, -CH2), 2.82 (s, 3H, -CH3), 2.63 (s, 6H, -CH3), 2.62 (s, 6H, -CH3), 1.80 (s, 1H, -OH). 13C-NMR (125MHz, CDCl3) (ppm): 164.7, 155.3, 152.4, 149.8, 149.6, 138.7, 136.7, 130.2, 122.0, 63.7, 38.8, 22.9, 22.4, 21.8. HRMS (ESI) m/z: $[M+H]^+$ calcd for $C_{16}H_{19}N_2O_3$: 287.13957, found: 287.13821.

*4-(hydroxymethyl)-2-methoxyphenyl 3,5,6-trimethylpyrazine-2-carboxylate (**6c**). White solid, m.p.: 142.3-143.4 °C, yield 35.2 %.* 1H-NMR (500MHz, CDCl3) (ppm): 7.16 (d, j = 8.0 Hz, 2H, Ar-H), 6.95 (d, j = 8.0 Hz, 2H, Ar-H), 4.70 (s, 2H, -CH2), 3.84 (s, 3H, -OCH3), 2.83 (s, 3H, -CH3), 2.62 (s, 3H, -CH3), 2.61 (s, 3H, -CH3), 2.04 (s, 1H, -OH). 13C-NMR (125MHz, CDCl3) (ppm): 164.1, 155.2, 152.4, 151.3, 149.8, 140.4, 139.3, 138.8, 123.0, 119.1, 111.1, 65.1, 56.0, 22.8, 22.4, 21.8. HRMS (ESI) m/z: $[M+H]^+$ calcd for $C_{16}H_{18}N_2O_4$: 303.134482, found: 303.13190.

*4-((3,5,6-trimethylpyrazine-2-carbonyl)oxy)benzyl 3,5,6-trimethylpyrazine-2-carboxylate (7**a**).* White solid, m.p.: 83.7-84.6 °C, yield 53.5 %. 1H-NMR (500MHz, CDCl3) (ppm): 7.56 (d, j = 8.2 Hz, 2H, Ar-H), 7.27 (d, j = 8.2 Hz, 2H, Ar-H), 5.46 (s, 2H, -CH2), 2.82 (s, 3H, -CH3), 2.73 (s, 3H, -CH3), 2.63 (s, 3H, -CH3), 2.62 (s, 3H, -CH3), 2.57 (s, 6H, -CH3). 13C-NMR (125MHz, CDCl3) (ppm): 165.8, 164.5, 155.5, 154.7, 152.6, 151.0, 149.8, 139.5, 138.4, 133.7, 130.0, 122.2, 66.8, 23.0, 22.7, 22.4, 22.3, 21.8, 21.7. HRMS (ESI) m/z: $[M+Na]^+$ calcd for $C_{23}H_{24}N_4NaO_4$: 443.16952, found: 443.16602.

*4-(2-((3,5,6-trimethylpyrazine-2-carbonyl)oxy)ethyl)phenyl 3,5,6-trimethylpyrazine-2-carboxylate (7**b**).* White solid, m.p.: 57.6-58.4 °C, yield 49.9 %. 1H-NMR (500MHz, CDCl3) (ppm): 7.35 (d, j = 8.2 Hz, 2H, Ar-H), 7.18 (d, j = 8.2 Hz, 2H, Ar-H), 4.61 (t, j = 7.2 Hz, 2H, -CH2), 3.14 (t, j = 7.2 Hz, 2H, -CH2),2.81 (s, 3H, -CH3), 2.68 (s, 3H, -CH3), 2.62 (s, 3H, -CH3), 2.61 (s, 3H, -CH3), 2.57 (brs, 6H, -CH3). 13C-NMR (125MHz, CDCl3) (ppm): 166.0, 164.6, 155.4, 154.6, 152.5, 151.3, 149.7, 149.6, 149.3, 139.6, 138.6, 135.5, 130.2, 122.0, 66.2, 34.7, 22.9, 22.4, 22.4, 22.3, 21.8, 21.7. HRMS (ESI) m/z: $[M+H]^+$ calcd for $C_{24}H_{27}N_4O_4$: 435.20323, found: 435.20108.

*2-methoxy-4-(((3,5,6-trimethylpyrazine-2-carbonyl)oxy)methyl)phenyl 3,5,6-trimethylpyrazine-2-carboxylate (7**c**).* White solid, m.p.: 101.9-102.8 °C, yield 39.5 %. 1H-NMR (500MHz, CDCl3) (ppm): 7.20 (d, j = 8.1 Hz, 1H, Ar-H), 7.15 (s, 1H, Ar-H), 7.10 (d, j = 8.1 Hz, 1H, Ar-H), 5.43 (s, 2H, -CH2), 3.84 (s, 3H, -CH3), 2.82 (s, 3H, -CH3), 2.73 (s, 3H, -CH3), 2.62 (s, 3H, -CH3), 2.60 (s, 3H, -CH3), 2.57 (s, 6H, -CH3). 13C-NMR (125MHz, CDCl3) (ppm): 165.8, 163.9, 155.3, 154.8, 151.2, 149.8, 139.9, 139.4, 138.5, 134.9, 123.1, 121.1, 112.7, 67.1, 56.0, 22.9, 22.8, 22.5, 22.3, 21.8, 21.8. HRMS (ESI) m/z: $[M+Na]^+$ calcd for $C_{24}H_{26}N_4NaO_5$: 473.18009, found: 473.17734.

Bio-Evaluation Methods

Protective effect of ligustrazine derivatives on damaged differentiated PC12 cells [3, 4]

PC12 cells were cultured in RPMI 1640 medium supplemented with 5 % (v/v) fetal bovine serum, 10 % (v/v) horse serum and 100 U/mL penicillin-streptomycin (Thermo Technologies, New York, NY, USA) at 37 °C in a humidified atmosphere of 5% CO_2. When cells achieved the desired density of >80 % confluency original medium was removed and cells were cultured with the serum-free medium for 14 h. Then the cells were suspended in 1640 medium supplemented with 10 % (v/v) fetal bovine serum, and seeded into Poly-L-lysine-coated 96-well culture plates at 7×10^3 cells/well, differentiated by treated with 50 ng/mL NGF for 48 h. After these, the differentiated PC12 cells were pretreated with various concentrations (60, 30, 15, 7.5, 3.75 μM) of ligustrazine derivatives for 36 h. All measurements were performed after the cells were induced by $CoCl_2$ (final concentration, 200 mM) for 12 h. Control differentiated cells were not treated with ligustrazine derivatives and $CoCl_2$. $CoCl_2$ was dissolved in RPMI 1640 medium. ligustrazine derivatives were dissolved in DMSO. The final concentration of DMSO was less than 0.1 % (v/v).

After MTT solution (20 μL, 5 mg/mL) was added to each well, the plate was incubated for a further 4 h at 37 °C. The supernatant was removed carefully by pipetting from wells without disturbing the attached cells and formazan crystals were solubilized by adding 200 μL of DMSO to each well and shaken for 15 min. The absorbance at 490 nm was measured with a BIORAD 550 spectrophotometer (Bio-rad, California, CA, USA). The proliferation rates of damaged PC12 cells were calculated by the formula $[OD_{490}\ (Compd) - OD_{490}\ (CoCl_2)]/[OD_{490}\ (NGF) - OD_{490}\ (CoCl_2)] \times 100\%$; the EC_{50} values were defined as the concentration of compounds that produced a 50% proliferation of surviving cells and calculated using the following equation: $-pEC_{50} = \log C_{max} - \log 2 \times (\sum P - 0.75 + 0.25P_{max} + 0.25P_{min})$, 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.

Observation of Morphologic Changes [5,6]

The PC12 cells culture procedure was similar to that described in section 3.2.1. After pretreatment with the serum-free medium for 14 h, cells were seeded at a concentration of 7×10^4 cell/mL in a volume of 0.8 mL on a Poly-L-lysine-coated sterile cover slip in 6-well tissue culture plates, and differentiated by treating with NGF (50 ng/mL) for 48 h. Then the differentiated PC12 cells were pretreated with compound **2c** (30 μM) for 36 h prior to exposure to $CoCl_2$. The cellular morphology and percentage of cells showing neurite outgrowth was determined by light microscopy (Nikon, Kobe, Japan). Cells with one or more neurites whose lengths were at least twice the diameter of the cell body were scored as differentiated cells. Cell differentiation rate was calculated as the number of differentiated cells/total cells. Neurite outgrowth was determined from at least three different regions of interest in three independent experiments. All data are expressed as mean ± SD. Data analysis was carried out using SAS software, version 8.1 (SAS Institute, Cary, NC, USA). Statistically significant differences between the samples were evaluated by Student's t-test and $p < 0.05$ was considered significant.

Plasma stability of 2c in vitro [7, 8]

2c and **T-VA** was dissolved in dimethylsulfoxide to make a 1 mg/ml solution, then attenuated by ethanol: water (1:1) solution to 100 μg/mL stock. Male Wistar rat plasma previously prepared and stored at − 80 °C was used to measure the plasma stability at six time points (0, 10, 30, 60, 120 and 240 min). Immediately before each experiment, suitable aliquots of the plasma were pre-incubated for 15 min at 37 °C. The experiments were initiated by adding the compounds solution to produce a final concentration of 5μg/mL. A 300 μL aliquot of methanol containing 0.2 % $HClO_4$ was added to 100 μL of sample solution immediately after it was taken from the reaction mixture, and cooled in an ice-bath. The mixture was stirred in a vortex mixer for 30 s and centrifuged at 3000 rpm at 4 °C for 10 min; 20 μL of the supernatant was injected into the chromatograph.

The separation and detection were carried out using a Waters e2695 high performance liquid chromatograph (Milford, MA, USA) consisted of Waters Alliance e2695 separation module and Waters 2489 UV detector. Chromatographic separations were performed with a Waters XBridge C18 (250 × 4.6 mm, 5 μm) column maintained at 30°C. The mobile phase was 0.05 % v/v formic acid in water/methyl alcohol with a constant flow rate of 1 mL/min. The methyl alcohol percentages were: 0–20 min, linearly from 60 % to 90 %; 20–25 min, 70 %. The degradation and residual percentages after 240 min incubation in rat plasma of **2c** and **T-VA** were showed in Table 1 and Table 2

Table 2. The analysis for **2c** degradation and residual percentages after 240 min incubation in rat plasma.

Time	Residue rate（%）
t_0	100
t_{10}	98.10±5.63
t_{30}	96.01±4.55
t_{60}	92.60±4.81
t_{120}	91.07±4.83
t_{240}	90.55±5.38

Table 3. The analysis for **T-VA** degradation and residual percentages after 240 min incubation in rat plasma.

Time	Residue rate（%）
t_0	100
t_{10}	64.86±3.53
t_{30}	60.68±4.71
t_{60}	40.41±4.18
t_{120}	26.78±5.16
t_{240}	5.37±4.69

Protective effect on injured neuronal-like PC12 cells

PC12 cells induced by nerve growth factor (NGF) bearing morphologically neurites and widely used in screening of neuroprotective agents. All the synthesized compounds were tested for their

protective effects on the neuronal-like PC12 cells damaged by $CoCl_2$, and TMP was used as the positive control drug. This revealed the proliferation rates (%) at different concentration and 50% effective concentrations (EC_{50}) for protecting damaged PC12 cells of the ligustrazine-phenols derivatives in Table 3. The results showed that TMP and its derivatives presented protective effects on injured differentiated PC12 cells and most of the ligustrazine derivatives were more active (with lower EC_{50} values) than TMP (EC_{50} = 64.46 μM).

Table 34. EC_{50} of the ligustrazine-phenols derivatives for protecting damaged PC12 cells

Compound	Proliferation rate (%)					EC_{50} (μM)
	3.75 μM	7.5 μM	15 μM	30 μM	60 μM	
1a	38.23	35.73	63.56	59.55	56.61	14.64
1b	36.46	29.53	44.10	57.06	69.41	16.49
1c	15.02	22.70	55.86	71.07	127.07	10.44
2a	47.56	82.12	119.96	162.07	128.12	1.66
2b	70.67	57.55	96.71	134.86	151.70	2.03
2c	72.05	85.86	109.63	138.95	129.99	1.07
3a	53.33	57.55	90.76	116.83	100.88	4.11
3b	38.47	46.97	33.75	64.21	28.61	19.76
3c	24.52	25.10	24.36	46.03	42.92	28.87
4a	31.06	38.37	37.24	64.61	64.13	16.72
4b	61.47	9.04	9.08	82.09	23.38	23.89
4c	11.62	8.73	25.93	43.60	33.83	39.09
5a	26.45	-4.60	22.75	42.63	49.66	37.18
5b	41.63	47.78	75.73	60.20	80.32	25.77
5c	-1.52	-3.63	33.31	22.79	15.93	60.29
6a	45.42	48.79	79.84	73.97	14.75	13.86
6b	13.53	-37.00	36.35	68.94	63.26	34.93
6c	35.45	56.91	76.89	74.59	84.59	13.87
7a	64.58	64.58	61.26	-4.83	-42.83	83.78
7b	24.71	18.28	16.08	40.85	32.26	36.57
7c	2.93	-39.51	-58.68	-65.27	-67.47	＞100
TMP	14.71	12.11	11.76	10.60	9.44	64.46

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