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Electronic Supplementary Information

Synthesis and protective effect of new ligustrazine derivatives against $CoCl_2$-induced neurotoxicity in differentiated PC12 cells. Part 2

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Bio-Evaluation Methods

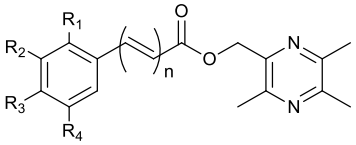
Protective effect on damaged differentiated PC12 cells [1, 2, 3]

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) at 37 ℃ 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. Thereafter, 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 0.5% (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 ℃. 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. The proliferation rates of damaged PC12 cells were calculated by [OD490 (Compd) − OD490 ($CoCl_2$)] / [OD490 (NGF) − OD490 ($CoCl_2$)] × 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.

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.

Table 1 The structures of ligustrazine derivatives **1-12**.



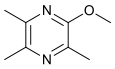
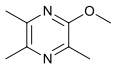
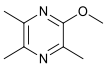
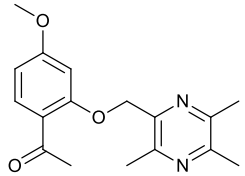
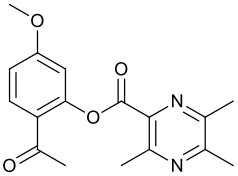
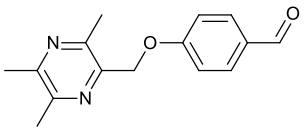
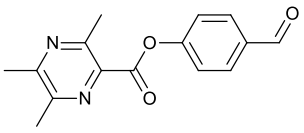
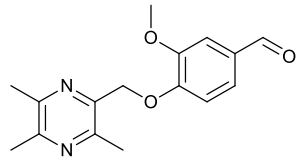
Compd	n	R_1	R_2	R_3	R_4	Yield
1	1	H	H	H	H	61.3%
2	1	H	H	CH_3	H	57.2%
3	1	OCH_3	H	H	H	53.7%
4	1	H	OCH_3	H	H	48.6%
5	1	H	H	OCH_3	H	51.1%
6	1	OCH_3	OCH_3	H	H	47.6%
7	1	H	OCH_3	OCH_3	H	46.9%
8	1	OCH_3	H	H	OCH_3	51.0%
9	1	OCH_3	OCH_3	OCH_3	H	48.3%
10	1	H	OCH_3	OCH_3	OCH_3	50.3%
11	0	$OOCCH_3$	H	H	H	58.3%
12	0	H				21.6%

Table 2 The structures of ligustrazine derivatives **13a-16a, 13b-16b**.

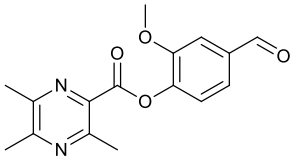
Structure	Yield	Structure	Yield
	52.6 %		52.1 %
13a		**13b**	
	42.6%		30.2%

14a



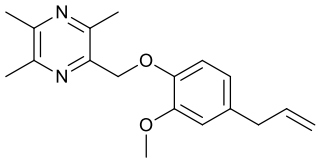
61.3 %

14b



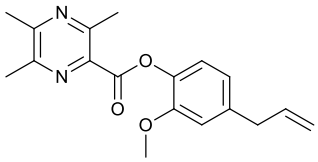
48.2 %

15a



59.1 %

15b



54.7 %

16a **16b**

Table 3 The EC_{50} values for protection on damaged PC12 cells of the ligustrazine derivatives.

Compd	Proliferation rate (%)					EC_{50}
	60 μM	30 μM	15 μM	7.5 μM	3.75 μM	(μM)
1	49.66	48.73	31.19	28.20	27.18	24.508
2	−19.30	−19.60	−49.29	−46.66	−83.92	>100
3	109.48	98.91	79.53	50.45	50.19	5.177
4	48.42	49.76	84.29	49.49	43.67	11.965
5	90.35	82.09	67.35	47.61	21.67	9.755
6	117.76	113.56	84.75	59.10	24.32	4.948
7	57.25	97.92	90.91	55.52	28.87	8.198
8	62.90	50.43	30.90	24.48	19.08	23.819
9	188.35	187.24	119.94	83.74	69.52	0.719
10	126.60	117.10	89.76	82.98	41.12	3.165
11	21.69	9.54	−0.24	−16.80	−17.45	102.458
12	−84.54	−85.47	−55.01	−40.63	−5.84	>100
13a	65.64	67.51	71.08	38.20	12.47	14.918
13b	19.00	33.61	20.24	14.41	4.85	49.854
14a	61.05	79.85	133.26	1.54	−14.72	12.159
14b	63.70	57.23	34.72	30.30	3.11	24.244
15a	18.84	21.49	53.51	20.94	2.52	40.611
15b	12.72	12.20	11.68	7.49	−13.47	81.717
16a	101.56	116.10	72.83	66.94	46.87	4.615
16b	4.60	14.82	10.63	8.53	−25.00	93.477
TMP	14.71	12.11	11.76	10.60	9.44	64.459

[a]EC_{50}: concentration of compound required to achieve 50% protection of PC12 cell from $CoCl_2$ induced neurotoxicity, as determined by the MTT method.

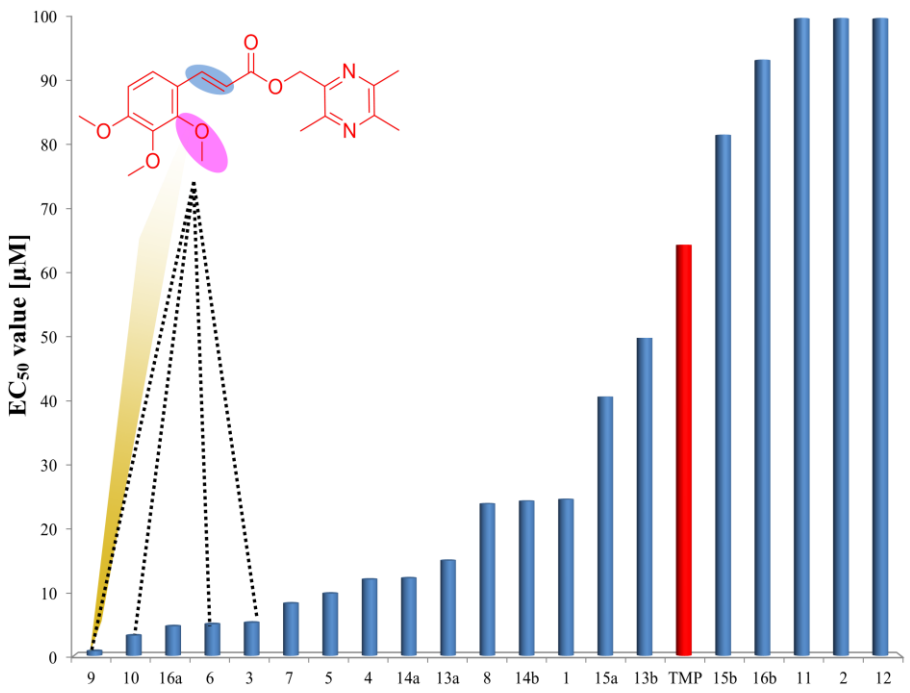


Figure 1 Structure-activity relationships of ligustrazine derivatives. The results showed that both olefinic group and o-methoxy substituent may contribute to enhance the efficacy of such newly ligustrazine derivatives.

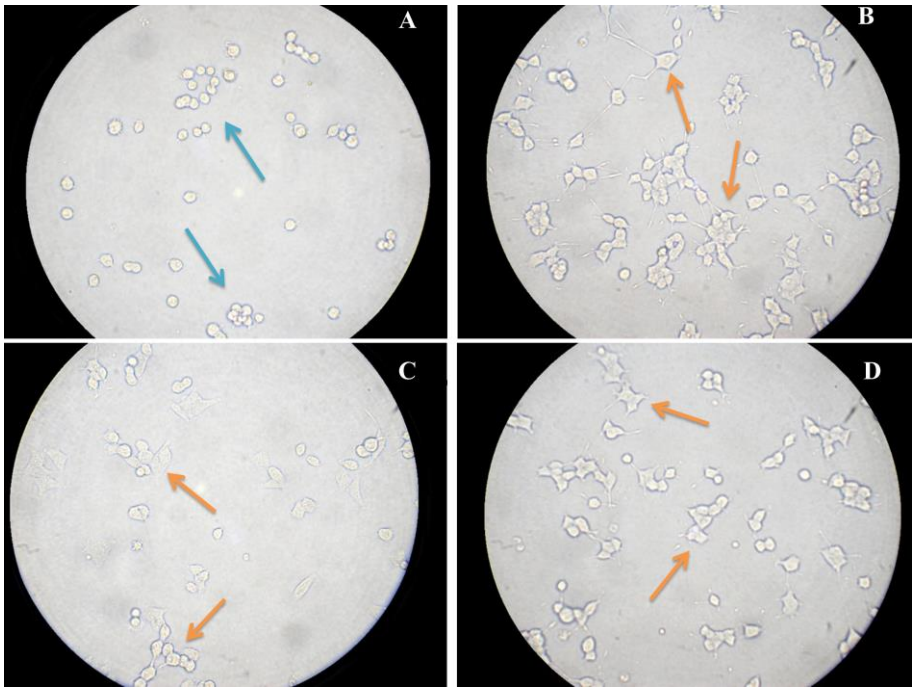


Figure 2 Protective effects of compound **9** against $CoCl_2$-induced injury in differentiated PC12 cells (×200). The most representative fields are shown. A: Undifferentiated PC12 cells. B: Differentiated PC12 cells by NGF. C: $CoCl_2$-induced neurotoxicity of differentiated PC12 cells. D: $CoCl_2$-induced neurotoxicity+ **9** (60μM)

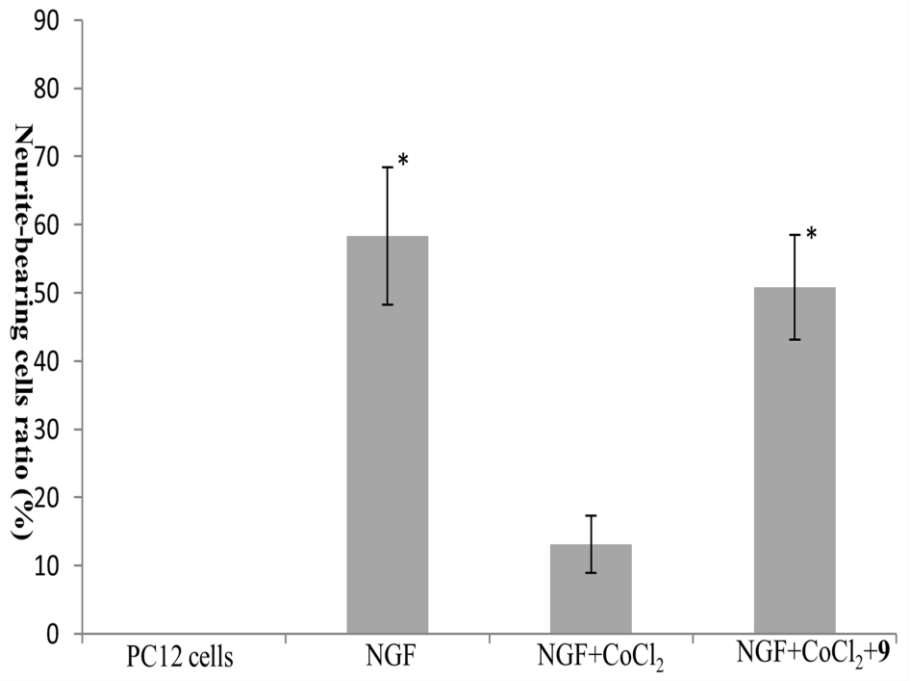
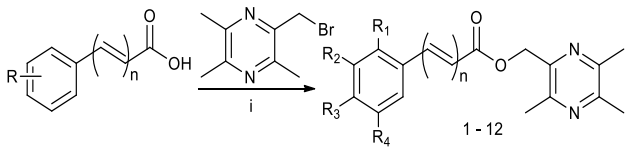


Figure 3 Protective effects of compound **9** (60μM) against $CoCl_2$-induced injury in differentiated PC12 cells. The neurite-bearing ration are shown as means ± S.D. of at least 3 independent experiments.*$p \leq 0.05$ level, significance relative to $CoCl_2$ group.

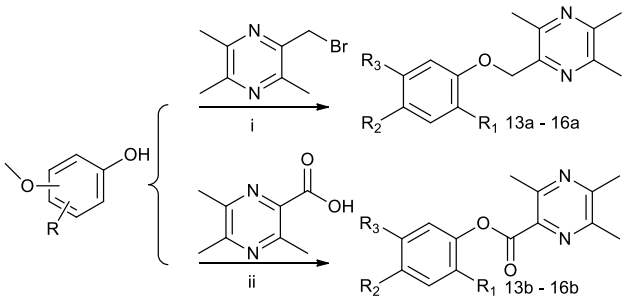
Synthesis

Materials and Methods

Reactions were monitored by TLC using silica gel coated aluminum sheets (Qingdao Haiyang Chemical Co., Qingdao, China) and visualized in UV light (254 nm). ^{1}H-NMR and ^{13}C-NMR assays were recorded on a BRUKER AVANCE 500 NMR spectrometer (Fällanden, Switzerland) and chemical shifts are reported in δ (ppm). HRMS spectra were obtained by using Thermo Scientific™ LTQ Orbitrap XL hybrid FTMS instrument (New York, NY, USA). Melting points (uncorrected) were measured on an X-5 micro melting point apparatus (Beijing, China). Flash column chromatography was performed using 200-300 mesh silica gel. The yields were calculated based on the last step reaction. All chemicals and solvents used were analytical or high-performance liquid chromatography grade. Concentration of the reaction solutions involved the use of a rotary evaporator under reduced pressure.



Scheme 1 Synthesis of ligustrazine derivatives (1–12). Reagents and Conditions: (i) DMF, K_2CO_3, 85 ℃, 1.5 h.



Scheme 2 Synthesis of ligustrazine derivatives (13–16). Reagents and Conditions: (i) DMF, K_2CO_3, 85 ℃, 1.5 h. (ii) CH_2Cl_2, EDCI/DMAP, r.t., 16 h.

2-(bromomethyl)-3,5,6-trimethylpyrazine. This intermediate was prepared according to our previous reported method [4]. The crude product, with 70% purity, was not purified further as it caused a strong mucous membrane irritation.

3,5,6-trimethylpyrazine-2-carboxylic acid. This intermediate was also prepared according to our previous reported method [1, 5]. The crude product was purified by recrystallization with acetone to obtain light yellow solid. Yield: 47.8%, m.p.: 162-163 ℃.

General procedure for the preparation of ligustrazine derivatives (**1**–**12**, **13a**–**16a**, method as shown in Scheme 1, 2).

2-(bromomethyl)-3,5,6-trimethylpyrazine (4.0 mmol) and aromatic acid derivatives (1.0 mmol) were dissolved in dry DMF, then K_2CO_3 (6.0 mmol) was added and the mixture was kept at 85 ℃ for 1.5 h under nitrogen atmosphere. The warm reaction mixture was poured into ice-water and 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.

(3,5,6-trimethylpyrazin-2-yl)methyl cinnamate (**1**). White solid, m.p.: 57.7–58.4 ℃. ^{1}H-NMR (500 MHz, $CDCl_3$, δ ppm): 7.74 (d, J = 16.0 Hz, 1H, -CH=), 7.54 (m, 2H, Ar-H), 7.41 (m, 3H, Ar-H), 6.51 (d, J = 16.0 Hz, 1H, =CH-), 5.36 (d, 2H, -C$\underline{H}_2$), 2.60 (s, 3H, -C$\underline{H}_3$), 2.55 (brs, 6H, -C$\underline{H}_3$). ^{13}C-NMR (500 MHz, $CDCl_3$, δ ppm): 166.6, 153.8, 151.3, 149.2, 149.1, 145.6, 145.0, 134.2, 130.5, 128.9, 128.2, 117.3, 65.2 (-$\underline{C}H_2$), 21.7 (-$\underline{C}H_3$), 21.5 (-$\underline{C}H_3$), 20.5 (-$\underline{C}H_3$). HRMS (ESI) *m/z*: 283.14389 $[M+H]^+$, calcd. for $C_{17}H_{19}N_2O_2$ 283.13683.

(*E*)-(3,5,6-trimethylpyrazin-2-yl)methyl 3-(p-tolyl)acrylate (**2**). White solid, m.p.: 71.7–72.5 ℃. ^{1}H-NMR (500 MHz, $CDCl_3$, δ ppm): 7.72 (d, J = 16.0 Hz, 1H, -CH=), 7.42 (d, J = 8.0 Hz, 2H, Ar-H), 7.20 (d, J = 8.0 Hz, 2H, Ar-H), 6.46 (d, J = 16.0 Hz, 1H, =CH-), 5.34 (s, 2H, -C$\underline{H}_2$), 2.59 (s, 3H, -C$\underline{H}_3$), 2.54 (s, 3H, -C$\underline{H}_3$), 2.38 (s, 3H, -C$\underline{H}_3$). ^{13}C-NMR (500 MHz, $CDCl_3$, δ ppm): 166.8, 151.3, 149.2, 149.1, 145.6, 145.0, 140.9, 131.5, 129.6, 128.2, 116.3, 65.2 (-$\underline{C}H_2$), 21.7 (-$\underline{C}H_3$), 21.5 (-$\underline{C}H_3$), 20.6 (-$\underline{C}H_3$). HRMS (ESI) *m/z*: 297.15997 $[M+H]^+$, calcd. for $C_{18}H_{21}N_2O_2$ 297.15248.

(*E*)-(3,5,6-trimethylpyrazin-2-yl)methyl 3-(2-methoxyphenyl)acrylate (**3**). White solid, m.p.: 41.8–42.6 ℃. ^{1}H-NMR (500 MHz, $CDCl_3$, δ ppm): 8.04 (d, J = 16.0 Hz, 1H, -CH=), 7.50 (d, J = 7.5 Hz, 1H, Ar-H), 7.36 (td, J = 7.75, 1.5 Hz, 1H, Ar-H), 6.96 (t, J = 7.5 Hz, 1H, Ar-H), 6.92 (d, J = 8.0 Hz, 1H, Ar-H), 6.60 (d, J = 16.0 Hz, 1H, =CH-), 5.35 (s, 2H, -C$\underline{H}_2$), 3.89 (s, 3H, -OC$\underline{H}_3$), 2.60 (s, 3H, -C$\underline{H}_3$), 2.54 (brs, 6H, -C$\underline{H}_3$). ^{13}C-NMR (500 MHz, $CDCl_3$, δ ppm): 167.2, 158.5, 151.2, 149.2, 149.0, 145.1, 141.1, 131.6, 129.2, 123.3, 120.7, 117.9, 111.1, 65.1 (-$\underline{C}H_2$), 55.5 (-O$\underline{C}H_2$), 21.7 (-$\underline{C}H_3$), 21.5 (-$\underline{C}H_3$), 20.6 (-$\underline{C}H_3$). HRMS (ESI) *m/z*: 313.15457 $[M+H]^+$, calcd. for $C_{18}H_{21}N_2O_3$ 313.14739.

(*E*)-(3,5,6-trimethylpyrazin-2-yl)methyl 3-(3-methoxyphenyl)acrylate (**4**). White solid, m.p.: 52.9–53.7 ℃. ^{1}H-NMR (500 MHz, $CDCl_3$, δ ppm): 7.70 (d, J = 16.0 Hz, 1H, -CH=), 7.29 (d, J = 8.5 Hz, 1H, Ar-H), 7.12 (d, J = 7.5 Hz, 1H, Ar-H), 7.04 (brs, 1H, Ar-H), 7.12 (dd, J = 8.0, 2.0 Hz, 1H, Ar-H), 6.49 (d, J = 16.0 Hz, 1H, =CH-), 5.35 (s, 2H, -C$\underline{H}_2$), 3.83 (s, 3H, -OC$\underline{H}_3$), 2.59 (s, 3H, -C$\underline{H}_3$), 2.54 (brs, 6H, -C$\underline{H}_3$). ^{13}C-NMR (500 MHz, $CDCl_3$, δ ppm): 166.5, 159.9, 151.4, 149.2, 145.5, 144.9, 135.6, 129.9, 120.9, 117.7, 116.4, 112.9, 65.2 (-$\underline{C}H_2$), 55.3 (-O$\underline{C}H_3$), 21.7 (-$\underline{C}H_3$), 21.5 (-$\underline{C}H_3$), 20.5 (-$\underline{C}H_3$). HRMS (ESI) *m/z*: 313.15482 $[M+H]^+$, calcd. for $C_{18}H_{21}N_2O_3$ 313.14739.

(*E*)-(3,5,6-trimethylpyrazin-2-yl)methyl 3-(4-methoxyphenyl)acrylate (**5**). White solid, m.p.: 71.9–72.8 ℃. ^{1}H-NMR (500 MHz, $CDCl_3$, δ ppm): 7.70 (d, J = 16.0 Hz, 1H, -CH=), 7.48 (d, J = 9.0 Hz, 2H, Ar-H), 6.91 (d, J = 8.5 Hz, 2H, Ar-H), 6.37 (d, J = 16.0 Hz, 1H, =CH-), 5.34 (s, 2H, -C$\underline{H}_2$), 3.85 (s, 3H, -OC$\underline{H}_3$), 2.59 (s, 3H, -C$\underline{H}_3$), 2.54 (brs, 6H, -C$\underline{H}_3$). ^{13}C-NMR (500 MHz, $CDCl_3$, δ ppm): 166.9, 161.5, 151.3, 149.2, 149.1, 145.2, 145.1, 129.8, 127.0, 114.8, 114.3, 65.1 (-$\underline{C}H_2$), 55.4 (-O$\underline{C}H_3$), 21.7 (-$\underline{C}H_3$), 21.5 (-$\underline{C}H_3$), 20.6 (-$\underline{C}H_3$). HRMS (ESI) *m/z*: 313.15512 $[M+H]^+$, calcd. for $C_{18}H_{21}N_2O_3$ 313.14739.

(*E*)-(3,5,6-trimethylpyrazin-2-yl)methyl 3-(2,3-dimethoxyphenyl)acrylate (**6**). White solid, m.p.: 84.2–84.9 ℃. ^{1}H-NMR (500 MHz, $CDCl_3$, δ ppm): 8.05 (d, J = 16.0 Hz, 1H, -CH=), 7.14 (d, J = 7.5 Hz, 1H, Ar-H), 7.06 (t, J = 8.0 Hz, 1H, Ar-H), 6.95 (d, J = 7.5 Hz, 1H, Ar-H), 6.55 (d, J = 16.0 Hz, 1H, =CH-), 5.35 (s, 2H, -C$\underline{H}_2$), 3.88 (s, 3H, -OC$\underline{H}_3$), 3.86 (s, 3H, -OC$\underline{H}_3$), 2.59 (s, 3H, -C$\underline{H}_3$), 2.53 (brs, 6H, -C$\underline{H}_3$). ^{13}C-NMR (500 MHz, $CDCl_3$, δ ppm): 166.8, 153.1, 151.3, 149.2, 149.0, 148.5, 145.0, 140.3, 128.5, 124.2, 119.3, 118.7, 114.1, 65.2 (-$\underline{C}H_2$), 61.3 (-O$\underline{C}H_3$), 55.9 (-O$\underline{C}H_3$), 21.7 (-$\underline{C}H_3$), 21.5 (-$\underline{C}H_3$), 20.6 (-$\underline{C}H_3$). HRMS (ESI) *m/z*: 343.16528 $[M+H]^+$, calcd. for $C_{19}H_{23}N_2O_4$ 343.15796.

(*E*)-(3,5,6-trimethylpyrazin-2-yl)methyl 3-(3,4-dimethoxyphenyl)acrylate (**7**). White solid, m.p.: 117.0–117.7 ℃. ^{1}H-NMR (500 MHz, $CDCl_3$, δ ppm): 7.66 (d, J = 16.0 Hz, 1H, -CH=), 7.09 (d, J = 8.5 Hz, 1H, Ar-H), 7.04 (brs, 1H, Ar-H), 6.86 (d, J = 8.5 Hz, 1H, Ar-H), 6.36 (d, J = 16.0 Hz, 1H, =CH-), 5.33 (s, 2H, -C$\underline{H}_2$), 3.91 (s, 3H, -OC$\underline{H}_3$), 3.90 (s, 3H, -OC$\underline{H}_3$), 2.58 (s, 3H, -C$\underline{H}_3$), 2.52 (brs, 6H, -C$\underline{H}_3$). ^{13}C-NMR (500 MHz, $CDCl_3$, δ ppm): 166.8, 151.3, 151.2, 149.2, 149.1, 145.4, 145.0, 127.2, 122.8, 115.0, 111.0, 109.5, 65.1 (-$\underline{C}H_2$), 56.0 (-O$\underline{C}H_3$), 55.0 (-O$\underline{C}H_3$), 21.7 (-$\underline{C}H_3$), 21.5 (-$\underline{C}H_3$), 20.6 (-$\underline{C}H_3$). HRMS (ESI) *m/z*: 343.16528 $[M+H]^+$, calcd. for $C_{19}H_{23}N_2O_4$ 343.15796.

(*E*)-(3,5,6-trimethylpyrazin-2-yl)methyl 3-(2,5-dimethoxyphenyl)acrylate (**8**). White solid, m.p.: 107.0–107.8 ℃. ^{1}H-NMR (500 MHz, $CDCl_3$, δ ppm): 8.02 (d, J = 16.0 Hz, 1H, -CH=), 7.04 (brs, 1H, Ar-H), 6.92 (dd, J = 9.0, 2.5 Hz, 1H, Ar-H), 6.85 (d, J = 9.0 Hz, 1H, Ar-H), 6.57 (d, J = 16.0 Hz, 1H, =CH-), 5.34 (s, 2H, -C$\underline{H}_2$), 3.84 (s, 3H, -OC$\underline{H}_3$), 3.79 (s, 3H, -OC$\underline{H}_3$), 2.59 (s, 3H, -C$\underline{H}_3$), 2.54 (brs, 6H, -C$\underline{H}_3$). ^{13}C-NMR (500 MHz, $CDCl_3$, δ ppm): 167.2, 153.4, 152.9, 151.3, 149.2, 149.0, 145.1, 140.8, 123.7, 118.1, 117.4, 113.3, 112.4, 65.2 (-$\underline{C}H_2$), 56.0 (-O$\underline{C}H_3$), 55.8 (-O$\underline{C}H_3$), 21.7 (-$\underline{C}H_3$), 21.5 (-$\underline{C}H_3$), 20.6 (-$\underline{C}H_3$). HRMS (ESI) *m/z*: 343.16592 $[M+H]^+$, calcd. for $C_{19}H_{23}N_2O_4$ 343.15796.

(*E*)-(3,5,6-trimethylpyrazin-2-yl)methyl 3-(2,3,4-trimethoxyphenyl)acrylate (**9**). White solid, m.p.: 73.4–74.2 ℃. ^{1}H-NMR (500 MHz, $CDCl_3$, δ ppm): 7.93 (d, J = 16.5 Hz, 1H, -CH=), 7.25 (d, J = 8.5 Hz, 1H, Ar-H), 6.69 (d, J = 9.0 Hz, 1H, Ar-H), 6.47 (d, J = 16.0 Hz, 1H, =CH-), 5.33 (s, 2H, -C$\underline{H}_2$), 3.91 (s, 3H, -OC$\underline{H}_3$), 3.90 (s, 3H, -OC$\underline{H}_3$), 3.87 (s, 3H, -OC$\underline{H}_3$), 2.59 (s, 3H, -C$\underline{H}_3$), 2.53 (brs, 6H, -C$\underline{H}_3$). ^{13}C-NMR (500 MHz, $CDCl_3$, δ ppm): 167.1, 155.7, 153.4, 151.2, 149.2, 149.0, 145.1, 142.3, 140.6, 123.3, 121.4, 116.2, 107.6, 65.1 (-$\underline{C}H_2$), 61.4 (-O$\underline{C}H_3$), 60.9 (-O$\underline{C}H_3$), 56.1 (-O$\underline{C}H_3$), 21.7 (-$\underline{C}H_3$), 21.5 (-$\underline{C}H_3$), 20.6 (-$\underline{C}H_3$). HRMS (ESI) *m/z*: 373.17603 $[M+H]^+$, calcd. for $C_{20}H_{25}N_2O_5$ 373.16852.

(*E*)-(3,5,6-trimethylpyrazin-2-yl)methyl 3-(3,4,5-trimethoxyphenyl)acrylate (**10**). White solid, m.p.: 93.1–93.8 ℃. ^{1}H-NMR (500 MHz, $CDCl_3$, δ ppm): 7.64 (d, J = 16.5 Hz, 1H, -CH=), 6.75 (s, 2H, Ar-H), 6.41 (d, J = 16.0 Hz, 1H, =CH-), 5.34 (s, 2H, -C$\underline{H}_2$), 3.88 (brs, 9H, -OC$\underline{H}_3$), 2.59 (s, 3H, -C$\underline{H}_3$), 2.53 (brs, 6H, -C$\underline{H}_3$). ^{13}C-NMR (500 MHz, $CDCl_3$, δ ppm): 166.5, 153.4, 151.3, 149.2, 149.1, 145.5, 145.0, 140.2, 129.7, 116.6, 105.3, 65.2 (-$\underline{C}H_2$), 61.0 (-O$\underline{C}H_3$), 56.1 (-O$\underline{C}H_3$), 21.6 (-$\underline{C}H_3$), 21.5 (-$\underline{C}H_3$), 20.5 (-$\underline{C}H_3$). HRMS (ESI) *m/z*: 373.17563 $[M+H]^+$, calcd. for $C_{20}H_{25}N_2O_5$ 373.16852.

(3,5,6-trimethylpyrazin-2-yl)methyl 2-acetoxybenzoate (**11**). White solid, m.p.: 85.4–86.0 ℃. ^{1}H-NMR (500 MHz, $CDCl_3$, δ ppm): 8.05 (dd, J = 7.5, 1.0 Hz, 1H, Ar-H), 7.47 (td, J = 8.5, 1.0 Hz, 1H, Ar-H), 7.31 (t, J = 7.5 Hz, 1H, Ar-H), 7.12 (d, J = 8.0 Hz, 1H, Ar-H), 5.41 (s, 2H, -C$\underline{H}_2$), 2.59 (s, 3H, -C$\underline{H}_3$), 2.55 (s, 3H, -C$\underline{H}_3$), 2.54 (s, 3H, -C$\underline{H}_3$), 2.22 (s, 3H, -C$\underline{H}_3$). ^{13}C-NMR (500 MHz, $CDCl_3$, δ ppm): 169.6, 164.1, 151.5, 150.8, 149.4, 149.1, 144.6, 134.1, 131.9, 126.0, 123.9, 122.9, 65.9 (-$\underline{C}H_2$), 21.7 (-$\underline{C}H_3$), 21.4 (-$\underline{C}H_3$), 20.8 (-$\underline{C}H_3$), 20.6 (-$\underline{C}H_3$). HRMS (ESI) *m/z*: 315.13470 $[M+H]^+$, calcd. for $C_{17}H_{18}N_2O_4$ 314.12666.

(3,5,6-trimethylpyrazin-2-yl)methyl 3,4,5-tris((3,5,6-trimethylpyrazin-2-yl)methoxy)benzoate (**12**). White solid, m.p.: 173.8–174.5 ℃. ^{1}H-NMR (500 MHz, $CDCl_3$, δ ppm): 7.55 (s, 2H, Ar-H), 5.46 (s, 2H, -C$\underline{H}_2$), 5.18 (brs, 4H, -C$\underline{H}_2$), 5.08 (s, 2H, -C$\underline{H}_2$), 2.62-2.31 (m, 36H, -C$\underline{H}_3$). ^{13}C-NMR (500 MHz, $CDCl_3$, δ ppm): 165.7, 152.5, 151.5, 151.3, 150.8, 150.2, 150.1, 149.4, 149.0, 148.5, 148.3, 146.0, 145.1, 144.8, 142.0, 125.2, 109.2, 74.3 (-$\underline{C}H_2$), 71.0 (-$\underline{C}H_2$), 66.1 (-$\underline{C}H_2$), 21.7 (-$\underline{C}H_3$), 21.7 (-$\underline{C}H_3$), 21.7 (-$\underline{C}H_3$), 21.5 (-$\underline{C}H_3$), 21.3 (-$\underline{C}H_3$), 21.3 (-$\underline{C}H_3$), 20.7 (-$\underline{C}H_3$), 20.2 (-$\underline{C}H_3$). MS (ESI) *m/z*: 273.0 $[M+H]^+$, HRMS (ESI) *m/z*: 707.36584 $[M+H]^+$, calcd. for $C_{39}H_{47}N_8O_5$ 707.35912.

1-(4-methoxy-2-((3,5,6-trimethylpyrazin-2-yl)methoxy)phenyl)ethanone (**13a**). White solid, m.p.: 100.2–100.9 ℃. ^{1}H-NMR (500 MHz, $CDCl_3$, δ ppm): 7.84 (d, J = 8.5 Hz, 1H, Ar-H), 6.74 (d, J = 2.0 Hz, 1H, Ar-H), 6.55 (dd, J = 8.5, 2.0 Hz, 1H, Ar-H), 5.26 (s, 2H, -C$\underline{H}_2$), 3.88 (s, 3H, -OC$\underline{H}_3$), 2.63 (s, 3H, -C$\underline{H}_3$), 2.56 (s, 3H, -C$\underline{H}_3$), 2.54 (s, 3H, -C$\underline{H}_3$), 2.51 (s, 3H, -C$\underline{H}_3$). ^{13}C-NMR (500 MHz, $CDCl_3$, δ ppm): 197.5, 164.4, 160.0, 151.7, 149.8, 148.8, 144.9, 132.7, 121.2, 106.1, 99.3, 70.2 (-$\underline{C}H_2$), 55.6 (-O$\underline{C}H_3$), 32.0 (-$\underline{C}H_3$), 21.7 (-$\underline{C}H_3$), 21.4 (-$\underline{C}H_3$), 20.6 (-$\underline{C}H_3$). HRMS (ESI) *m/z*: 301.15482 $[M+H]^+$, calcd. for $C_{17}H_{21}N_2O_3$ 301.14739.

4-((3,5,6-trimethylpyrazin-2-yl)methoxy)benzaldehyde (**14a**). White solid, m.p.: 121.0–121.7 ℃. ^{1}H-NMR (500 MHz, $CDCl_3$, δ ppm): 9.87 (s, 1H, -C$\underline{H}$O), 7.82 (d, J = 8.5 Hz, 2H, Ar-H), 7.11 (d, J = 8.5 Hz, 2H, Ar-H), 5.23 (s, 2H, -C$\underline{H}_2$), 2.57 (s, 3H, -C$\underline{H}_3$), 2.52 (brs, 6H, -C$\underline{H}_3$). ^{13}C-NMR (500 MHz, $CDCl_3$, δ ppm): 190.8, 163.5, 151.7, 150.0, 148.8, 144.8, 132.0, 130.3, 115.1, 70.1 (-$\underline{C}H_2$), 21.7 (-$\underline{C}H_3$), 21.4 (-$\underline{C}H_3$), 20.6 (-$\underline{C}H_3$). HRMS (ESI) *m/z*: 257.12888 $[M+H]^+$, calcd. for $C_{15}H_{16}N_2O_2$ 256.12118.

3-methoxy-4-((3,5,6-trimethylpyrazin-2-yl)methoxy)benzaldehyde (**15a**). White solid, m.p.: 119.0–119.7 ℃. ^{1}H-NMR (500 MHz, $CDCl_3$, δ ppm): 9.85 (s, 1H, -C$\underline{H}$O), 7.43 (m, 2H, Ar-H), 7.18 (d, J = 8.0 Hz, 1H, Ar-H), 5.30 (s, 2H, -C$\underline{H}_2$), 3.91 (s, 3H, -OC$\underline{H}_3$), 2.62 (s, 3H, -C$\underline{H}_3$), 2.52 (s, 3H, -C$\underline{H}_3$), 2.50 (s, 3H, -C$\underline{H}_3$). ^{13}C-NMR (500 MHz, $CDCl_3$, δ ppm): 190.9, 153.4, 151.6, 150.1, 150.1, 148.7, 144.8, 130.5, 126.5, 112.6, 109.3, 70.8 (-$\underline{C}H_2$), 56.0 (-O$\underline{C}H_3$), 21.7 (-$\underline{C}H_3$), 21.4 (-$\underline{C}H_3$), 20.7 (-$\underline{C}H_3$). HRMS (ESI) *m/z*: 287.13901 $[M+H]^+$, calcd. for $C_{16}H_{19}N_2O_3$ 287.13174.

2-((4-allyl-2-methoxyphenoxy)methyl)-3,5,6-trimethylpyrazine (**16a**). White solid, m.p.: 78.5–79.3 ℃. ^{1}H-NMR (500 MHz, $CDCl_3$, δ ppm): 6.96 (d, J = 7.8 Hz, 1H, Ar-H), 6.72 (brs, 1H, Ar-H), 6.69 (d, J = 7.8 Hz, 1H, Ar-H), 5.95 (m, 1H, -C$\underline{H}$=), 5.17 (s, 2H, -C$\underline{H}_2$), 5.07 (m, 2H, =C$\underline{H}_2$), 3.83 (s, 3H, -OC$\underline{H}_3$), 3.33 (d, J = 6.5 Hz, 1H, -C$\underline{H}_2$), 2.62 (s, 3H, -C$\underline{H}_3$), 2.50 (brs, 6H, -C$\underline{H}_3$). ^{13}C-NMR (500 MHz, $CDCl_3$, δ ppm): 151.0, 150.2, 149.8, 148.4, 146.4, 146.0, 137.6, 133.8, 120.4, 115.7, 114.8, 112.4, 71.3 (-$\underline{C}H_2$), 55.9, 39.8, 21.6 (-$\underline{C}H_3$), 21.4 (-$\underline{C}H_3$), 20.7 (-$\underline{C}H_3$). HRMS (ESI) *m/z*: 299.17548 $[M+H]^+$, calcd. for $C_{18}H_{23}N_2O_2$ 299.16813.

General procedure for the preparation of ligustrazine derivatives (**13b**–**16b**, method as shown in Scheme 2).

To a solution of *3,5,6-trimethylpyrazine-2-carboxylic acid* (1.0 mmol) in CH_2Cl_2 (30 mL) were successively added aromatic phenolic derivatives (1.0 mmol), EDCI (1.5 mmol), and DMAP (catalyst), and the mixture was stirred at

room temperature for 16 h under nitrogen atmosphere. The organic layer was washed with brine and water, respectively. 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 the target compound was recrystallized from acetone.

2-acetyl-5-methoxyphenyl 3,5,6-trimethylpyrazine-2-carboxylate (**13b**). White solid, m.p.: 134.2–134.8 ℃. ^{1}H-NMR (500 MHz, $CDCl_3$, δ ppm): 7.89 (d, J = 8.5 Hz, 1H, Ar-H), 6.87 (dd, J = 8.5, 2.5 Hz, 1H, Ar-H), 6.80 (d, J = 2.5 Hz, 1H, Ar-H), 3.87 (s, 3H, -OC$\underline{H}_3$), 2.85 (s, 3H, -C$\underline{H}_3$), 2.62 (s, 3H, -C$\underline{H}_3$), 2.61 (s, 3H, -C$\underline{H}_3$), 2.53 (s, 3H, -C$\underline{H}_3$). ^{13}C-NMR (500 MHz, $CDCl_3$, δ ppm): 195.7, 164.2, 163.8, 155.4, 153.2, 151.7, 149.5, 138.0, 132.6, 123.0, 112.0, 109.4, 55.8 (-O$\underline{C}H_3$), 29.2 (-$\underline{C}H_3$), 22.9 (-$\underline{C}H_3$), 22.3 (-$\underline{C}H_3$), 21.7 (-$\underline{C}H_3$). HRMS (ESI) *m/z*: 315.13419 $[M+H]^+$, calcd. for $C_{17}H_{19}N_2O_4$ 315.12666.

4-formylphenyl 3,5,6-trimethylpyrazine-2-carboxylate (**14b**). White solid, m.p.: 105.5–106.4 ℃. ^{1}H-NMR (500 MHz, $CDCl_3$, δ ppm): 10.05 (s, 1H, -C$\underline{H}$O), 8.01 (d, J = 8.5 Hz, 2H, Ar-H), 7.46 (d, J = 8.5 Hz, 2H, Ar-H), 2.86 (s, 3H, -C$\underline{H}_3$), 2.65 (brs, 6H, -C$\underline{H}_3$). ^{13}C-NMR (500 MHz, $CDCl_3$, δ ppm): 191.0, 163.8, 155.8, 155.6, 153.0, 149.8, 137.6, 134.3, 131.3, 122.8, 22.9 (-$\underline{C}H_3$), 22.4 (-$\underline{C}H_3$), 21.7 (-$\underline{C}H_3$). HRMS (ESI) *m/z*: 271.10733 $[M+H]^+$, calcd. for $C_{15}H_{14}N_2O_3$ 270.10044.

4-formyl-2-methoxyphenyl 3,5,6-trimethylpyrazine-2-carboxylate (**15b**). White solid, m.p.: 135.7–136.4 ℃. ^{1}H-NMR (500 MHz, $CDCl_3$, δ ppm): 9.99 (s, 1H, -C$\underline{H}$O), 7.54 (m, 2H, Ar-H), 7.40 (d, J = 7.5 Hz, 1H, Ar-H), 3.92 (s, 3H, -OC$\underline{H}_3$), 2.85 (s, 3H, -C$\underline{H}_3$), 2.64 (s, 3H, -C$\underline{H}_3$), 2.63 (s, 3H, -C$\underline{H}_3$). ^{13}C-NMR (500 MHz, $CDCl_3$, δ ppm): 191.1, 163.2, 155.6, 152.8, 152.0, 149.7, 145.1, 137.8, 135.5, 124.8, 123.6, 110.8, 56.1 (-O$\underline{C}H_3$), 22.8 (-$\underline{C}H_3$), 22.4 (-$\underline{C}H_3$), 21.7 (-$\underline{C}H_3$). HRMS (ESI) *m/z*: 301.11792 $[M+H]^+$, calcd. for $C_{16}H_{17}N_2O_4$ 301.11101.

4-allyl-2-methoxyphenyl 3,5,6-trimethylpyrazine-2-carboxylate (**16b**). White solid, m.p.: 103.2–103.8 ℃. ^{1}H-NMR (500 MHz, $CDCl_3$, δ ppm): 7.13 (d, J = 8.0 Hz, 1H, Ar-H), 6.84 (m, 2H, Ar-H), 5.99 (m, 1H, -C$\underline{H}$=), 5.12 (m, 2H, =CH_2), 3.84 (s, 3H, -OCH_3), 3.42 (d, J = 6.5 Hz, 1H, -CH_2), 2.85 (s, 3H, -C$\underline{H}_3$), 2.64 (s, 3H, -C$\underline{H}_3$), 2.62 (s, 3H, -C$\underline{H}_3$). ^{13}C-NMR (500 MHz, $CDCl_3$, δ ppm): 164.1, 154.9, 152.2, 150.8, 149.5, 139.3, 138.8, 138.0, 137.1, 122.6, 120.7, 116.2, 112.7, 55.8, 40.1, 22.7 (-$\underline{C}H_3$), 22.3 (-$\underline{C}H_3$), 21.7 (-$\underline{C}H_3$). HRMS (ESI) *m/z*: 313.15497 $[M+H]^+$, calcd. for $C_{18}H_{21}N_2O_3$ 313.14739.

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