Neurochemistry International 139 (2020) 104807

Contents lists available at ScienceDirect

Neurochemistry International

journal homepage: www.elsevier.com/locate/neuint

Highly efficient synthesis and monoamine oxidase B inhibitory profile of demethyleneberberine, columbamine and palmatine

Cheng Tao [a,b,1], Sheng-quan Hu [b,1], Jian Chen [a,b,1], Yuan-ji Chen [c], Ke-huan Sun [b], Guo-zhen Cui [d,**], Min Ma [e,f,a,***], Zheng-zhi Wu [b,g,1,*]

[a] Integrated Chinese and Western Medicine Postdoctoral Research Station, Jinan University, Guangzhou, China
[b] Shenzhen Institute of Geriatrics, Shenzhen Second People's Hospital, The First Affiliated Hospital of Shenzhen University, Shenzhen, China
[c] Dongguan Institute of Jinan University, Dongguan, China
[d] Department of Bioengineering, Zunyi Medical University Zhuhai Campus, Zhuhai, China
[e] College of Traditional Chinese Medicine, Jinan University, Guangzhou, China
[f] The First Affiliated Hospital of Jinan University, Guangzhou, China
[g] The Eighth Affiliated Hospital of Sun Yat-sen University, Shenzhen, China

ARTICLE INFO

Keywords:
Demethyleneberberine
Columbamine
Monoamine oxidase B
Reversibility

ABSTRACT

The biosynthesis of berberine alkaloids is thought to begin with the demethylation of berberine followed by methylation reactions to generate other type berberine alkaloids. This seemingly expeditious way to access berberine alkaloids has been stagnated for over half a century due to certain vexing synthetic problems, such as low isolated yield, complex operations and toxic reagents. We further investigated this bioinspired semi-synthesis strategy and significantly improved the synthetic efficacy, by providing a practical synthetic process for demethyleneberberine (DMB), columbamine and palmatine. Furthermore, we found that DMB (IC_{50}, 9.06 μM) inhibited the activity of monoamine oxidase B (MAO-B), an enzyme that deaminates dopamine and is particularly involved in the pathology of Parkinson's disease. Besides, columbamine was able to decrease MAO-B activity by approximately 40%. These findings provide perquisites for further *in vivo* investigation to confirm the therapeutic potentiality of berberine alkaloids, DMB in particular.

1. Introduction

Parkinson's disease (PD) is a progressive neurodegenerative condition that primarily damages the functional dopaminergic neurons in substantia nigra. Though the exact cause of PD still remains unclear, the reduction of dopamine level in striatum contributes largely to PD pathology. There is substantial evidence suggesting that dopamine is a substrate for type B monoamine oxidase (MAO-B) and that MAO-B inhibitors could exert therapeutic significance for treating PD by increasing dopamine amount (Tao et al., 2019; Tzvetkov et al., 2017). Thus, great effort has been placed on the search of MAO-B inhibitory small molecules.

Berberine alkaloids (Fig. 1) are a group of architecturally diverse protoberberine compounds that mainly include berberine, palmatine, jatrorrhizine, demethyleneberberine (DMB) and columbamine. Structurally, berberine alkaloids are quaternary ammonium salts and share a congested tetracyclic skeleton possessing hydroxyl oxidation state at C2, C3, C9 and C10 positions respectively. Notably, the presence of nitrogen at the intriguing conjugated molecular architecture was found to be associated with the pharmacological activities of cholesterol-lowering and anticancer effects (Wang et al., 2019). Additionally, various pharmacological activities on central nervous system provided by berberine analogues have been revealed in the past few years. For example, berberine was found to alleviate ischemic arrhythmias (Wang et al., 2012a), mitigate cognitive decline in a triple-transgenic mouse model of Alzheimer's disease (Chen et al., 2020), and ameliorate lipopolysaccharide-induced inflammatory response (Kim et al., 2019). Palmatine has been approved as an anti-inflammatory drug for clinical

* Corresponding author. Shenzhen Institute of Geriatrics, Shenzhen Second People's Hospital, The First Affiliated Hospital of Shenzhen University, Shenzhen, China
** Corresponding author.
*** Corresponding author. College of Traditional Chinese Medicine, Jinan University, Guangzhou, China.
E-mail address: szwzz001@email.szu.edu.cn (Z.-z. Wu).
[1] Cheng Tao, Sheng-quan Hu and Jian Chen contributed equally to this work.

https://doi.org/10.1016/j.neuint.2020.104807
Received 30 January 2020; Received in revised form 6 July 2020; Accepted 7 July 2020
Available online 22 July 2020

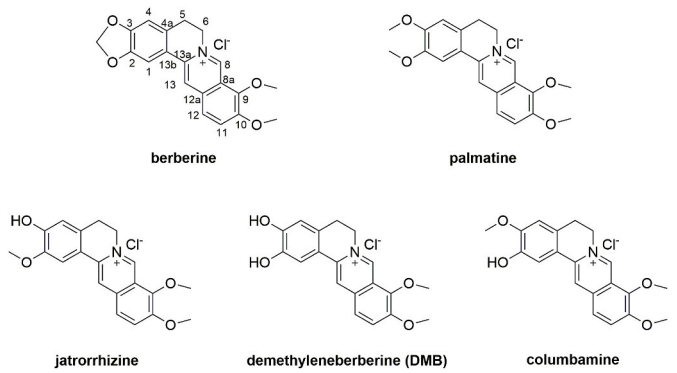


Fig. 1. The chemical structures of representative berberine alkaloids.

treatment of gastrointestinal infections, surgical infections and gynecological inflammation (Tarabasz and Kukula-Koch, 2019). More importantly, there is evidence suggesting that jatrorrhizine, but not berberine and palmatine, effectively inhibited the activity of MAO-B enzyme isolated from rat brain mitochondria (Kong et al., 2001), yet other studies showed that berberine weakly inactivated MAO-B that was purified from mouse liver mitochondria (Carradori et al., 2014; Castillo et al., 2005). This discrepancy might be explained by the use of MAO-B enzyme from different animal sources. Notably, the MAO-B inhibitory effects of other berberine alkaloids have not been systematically investigated yet.

The favorable bioactivities coupled with interesting molecular architecture rendered berberine alkaloids as an attractive synthetic target. During the past decades, berberine alkaloids have garnered significant attention from a synthetic perspective and many strategies have been employed for the total synthesis of such kind of natural products (Hanaoka et al., 2000; Orejarena Pacheco et al., 2013; Orito et al., 1999; Reddy et al., 2015; Wang et al., 2012b). However, most of these methods suffer from the multiple-step operations and harsh conditions, which impede the application of the current methodologies to multigram-scale production. Up to date, none of these reported methods has been applied for the industrial large-scale production and berberine alkaloids are mainly obtained from the isolation of natural source. The limited supply led to the high selling price of many commercial available berberine alkaloids, such as DMB ($300/10 mg, Energy) and columbamine ($340/10 mg, Mce), which underlines the necessity of a sufficient synthetic supply to meet the requirement of pharmacological research.

As depicted in Fig. 2, DMB, one of the major metabolites of berberine, has been considered as the central intermediate in the biogenetic pathway of berberine alkaloids. The biosynthesis of berberine alkaloids was thought to begin with the formation of DMB from berberine and then DMB underwent methylation to generate other type berberine alkaloids (M. Rueffer and Zenk, 1986). From a perspective of synthetic application, choosing the cheap berberine ($100/kg, Guide) as the starting material and undertaking a semi-synthesis in this biogenetic pathway would be the most rapid and practical way for the berberine alkaloids. In 1967, Cava MP first validated this biogenetic pathway and realized the synthesis of DMB and columbamine (Cava and Reed, 1967). However, the low isolated yield coupled with the use of volatile and toxic reagents make this strategy less effective and unpractical. It should be noted that these two seemingly simple transformations (demethylation and methylation) turned out to be problematic and still haven't been improved during the following 50 years. The significant difference of selling price between berberine ($100/kg, Guide), DMB ($300/10 mg, Energy) and columbamine ($340/10 mg, Mce) also illustrated this point, which promoted us to further explore this bio-inspired semi-synthesis strategy of berberine alkaloids. Herein, we reported a highly efficient and practical synthetic protocol for berberine alkaloids DMB, columbamine and palmatine and further investigated the MAO-B inhibitory effects of these alkaloids.

2. Materials and methods

MAO-B Inhibitor Screening Kit (#K797-100) was purchased from Biovision. Human MAO-B enzyme and tyramine hydrochloride were obtained from Sigma-Aldrich. Pierce™ horseradish peroxidase and Slide-A-Lyzer MINI Dialysis Device were from Thermo Scienific™. Amplex Red reagent was from Life Technologies.

2.1. General experimental

NMR spectra were recorded on (^{1}H at 400 MHz and ^{13}C at 100 MHz) spectrometers. Chemical shifts (δ) were given in ppm with reference to solvent signals [^{1}H NMR: DMSO-d_6 (2.50); ^{13}C NMR: DMSO-d_6 (39.52)]. Data for ^{1}H NMR spectra were reported as following: chemical shift (δ/ppm), multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, br = broad.), coupling constant (J/Hz) and integration. Data for ^{13}C NMR spectra were reported in terms of the chemical shift. High-resolution mass spectrometry (HRMS) was conducted on Bruker Apex IV RTMS. Flash column chromatography was performed on Tsingdao silica gel (60, particle size 0.040–0.063 mm). Thin-layer chromatography (TLC) was carried out on 0.25 mm Huanghai silica gel plates (HSGF-254) and visualized by exposure to UV light (254 nm) and/or $KMnO_4$ (aq.) was used as revealing system. All the solvents and reagents were used as obtained from commercial sources without further purification. Final products were of >98% purity as analyzed by HPLC analysis (Phenomenon luna C18, 5.0 μm, 250 mm*4.6 mm) on Thermo Ultimate 3000. The signal was monitored at 264 nm with a UV dector. The sample injection was 20 μL. A flow rate of 1.0 mL/min was used and the mobile phases consisted of solvent A (0.02 M, pH = 4, aq. KH_2PO_4) and solvent B (MeCN). The gradient elution was as follows: 0–1 min, 90% A and 10% B, *v/v*; 1–20 min, the volume fraction of B was increased to 90% at a constant rate; 20–29 min, 10% A and 90% B *v/v*; 29–30 min, the volume fraction of B was decreased to 10% at a constant rate; 30–40 min, 90% A and 10% B, *v/v*. Characterization data can be found in the Supplementary Data.

2.2. Synthesis of DMB

The reaction was carried out in mechanically stirred two-neck flask. To a stirred suspension of berberine (200 g, 0.54 mol) in xylene (1.3 L) was added trifluoromethanesulfonic acid (TfOH) (280 mL, 3.17 mmol), and the reaction mixture was stirred at room temperature for 30 min. Then 1 M HCl was added to the resulting mixture at 0 °C until the precipitate was not generated. The mixture was filtered and the precipitate was washed with water and petroleum ether. The resulting precipitate was re-crystallized with EtOH, which was converted to the

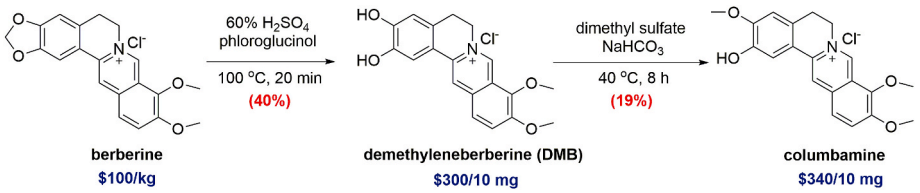


Fig. 2. Previous semi-synthesis of DMB and columbamine.

chloride by using anion-exchange resin (Cl^-). The resulting solution was concentrated in vacuo to afford DMB (174 g, 90%) as a yellow powder. R_f = 0.4 (DCM/MeOH, 10:1); mp: 220–222 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.04 (s, 1H, OH-2), 9.81 (s, 1H, H-8), 9.26 (s, 1H, OH-3), 8.74 (s, 1H, H-13), 8.16 (d, J = 9.1 Hz, 1H, H-11), 8.04 (d, J = 9.1 Hz, 1H, H-12), 7.50 (s, 1H, H-1), 6.80 (s, 1H, H-4), 4.88 (t, J = 6.3 Hz, 2H, H-6), 4.08 (s, 3H, OCH_3-10), 4.05 (s, 3H, OCH_3-9), 3.11 (t, J = 6.3 Hz, 2H, H-5); ^{13}C NMR (100 MHz, DMSO) δ 150.0 (C-10), 149.2 (C-12), 145.6 (C-9), 145.1 (C-3), 143.5 (C-8), 138.3 (C-13a), 133.3 (C-12a), 127.2 (C-4a), 126.7 (C-12), 123.5 (C-11), 121.2 (C-13b), 119.1 (C-8a), 117.8 (C-13), 114.9 (C-4), 112.7 (C-1), 61.9 (OCH_3-10), 57.1 (C-6), 55.6 (OCH_3-9), 25.8 (C-5); HRMS (ESI): m/z calcd for $C_{19}H_{18}NO_4Cl$ $[M\text{-}Cl]^+$: 324.1230, found: 324.1239; HPLC purity (99.40%). The spectral data for synthetic DMB (^{1}H NMR, ^{13}C NMR and HMRS) was consistent with earlier studies (Li et al., 2010; Spinozzi et al., 2014).

2.3. Synthesis of columbamine

To a stirred solution of DMB (2.5 g, 6.98 mmol) in MeCN (250 mL) was added K_2CO_3 (0.96 g, 6.98 mmol). Then methyl *p*-toluenesulfonate (TsOMe) (1.15 mL, 7.68 mmol) was slowly added dropwise by using the automatic injection pump through 6 h at 60 °C, and the reaction was monitored by TLC. After completed, the reaction mixture was cooled to room temperature and concentrated in vacuo. The resulting precipitate was converted to the chloride by using anion-exchange resin (Cl^-). The resulting solution was concentrated in vacuo and then the residue was purified by flash chromatography to afford columbamine (1.6 g, 60%) as a yellow powder. R_f = 0.6 (DCM/MeOH, 10:1); mp: 210–212 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.88 (s, 1H, H-8), 9.48 (s, 1H, H-13), 8.81 (s, 1H, OH-2), 8.19 (d, J = 9.1 Hz, 1H, H-12), 8.07 (d, J = 9.1 Hz, 1H, H-11), 7.58 (s, 1H, H-1), 7.06 (s, 1H, H-4), 4.94 (t, J = 6.1 Hz, 2H, H-6), 4.10 (s, 3H, OCH_3-10), 4.07 (s, 3H, OCH_3-9), 3.89 (s, 3H, OCH_3-3), 3.20 (t, J = 6.1 Hz, 2H, H-5);^{13}C NMR (100 MHz, DMSO) δ 150.6(C-2), 150.2 (C-3), 146.4 (C-9), 145.3 (C-10), 143.5 (C-8) 137.8 (C-4a), 133.1 (C-8a), 127.0 (C-12), 126.7 (C-11), 123.5 (C-12a), 121.3 (C-13a), 119.5 (C-13), 119.1 (C-13b), 112.2 (C-4), 111.4 (C-1), 61.9 (OCH_3-10), 57.1 (OCH_3-9), 55.9 (OCH_3-3), 55.5 (C-6), 26.0 (C-5); HRMS (ESI): m/z calcd for $C_{20}H_{20}NO_4Cl$ $[M\text{-}Cl]^+$: 338.1387, found: 338.1377; HPLC purity (98.55%). The spectral data for synthetic columbamine (^{1}H NMR, ^{13}C NMR, DEPT, COSY, HMBC, HMQC and HMRS) was consistent with earlier studies (Tong et al., 2005; Yu et al., 2011).

2.4. Synthesis of palmatine

To a stirred solution of DMB (10 g, 27.9 mmol) in MeCN (280 mL) was added K_2CO_3 (8.45 g, 61.4 mmol). Then methyl p-toluenesulfonate (TsOMe) (12.5 mL, 83.7 mmol) was slowly added. The reaction was stirred for 12 h at 80 °C, and the reaction was monitored by TLC. After DMB was fully consumed, the reaction mixture was cooled to room temperature and concentrated in vacuo. The resulting precipitate was washed with water and petroleum ether. The resulting precipitate was re-crystallized with EtOH, which was converted to the chloride by using anion-exchange resin (Cl^-). The resulting solution was concentrated in vacuo to afford palmatine (8.62 g, 80%) as a yellow powder. R_f = 0.7 (DCM/MeOH, 10:1); mp: 203–205 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.90 (s, 1H, H-8), 9.16 (s, 1H, H-13), 8.15 (d, J = 9.2 Hz, 1H, H-12), 8.04 (d, J = 9.1 Hz, 1H, H-11), 7.70 (s, 1H, H-1), 7.06 (s, 1H, H-4, H-4), 4.97 (t, J = 6.3 Hz, 2H, H-6), 4.09 (s, 3H, OCH_3-10), 4.05 (s, 3H, OCH_3-9), 3.92 (s, 3H, OCH_3-3), 3.85 (s, 3H, OCH_3-2), 3.22 (t, J = 6.4 Hz, 2H, H-5); ^{13}C NMR (100 MHz, DMSO) δ 151.4 (C-2), 150.2 (C-3), 148.7 (C-9), 145.3 (C-10), 143.6 (C-8), 137.5 (C-4a), 133.1 (C-8a), 128.4 (C-12), 126.6 (C-11), 123.5 (C-12a), 121.3 (C-13a), 120.0 (C-13), 118.9 (C-13b), 111.2 (C-4), 108.8 (C-1), 61.9 (OCH_3-10) 57.0 (OCH_3-9), 56.3 (OCH_3-3), 55.8 (OCH_3-2), 55.3 (C-6), 26.0 (C-5); RMS (ESI): m/z calcd for $C_{21}H_{20}NO_4Cl$ $[M\text{-}Cl]^+$: 352.1543, found: 352.1527; HPLC purity (99.56%). The spectral data for synthetic palmatine (^{1}H NMR, ^{13}C NMR and HMRS) was identical with an earlier study (Reddy et al., 2015).

2.5. MAO-B enzyme activity assay

MAO-B inhibitory effects of tested compounds were carried out in 96-well black plates using the commercial MAO-B Inhibitor Screening Kit. Briefly, 10 μl tested compounds (10 ×) were incubated with 50 μl MAO-B enzyme working solution for 30 min at 37 °C, and then 40 μl MAO-B substrate solution (MAO-B substrate, Developer and OxiRed™ Probe) was added into each well. After that, fluorescence (Ex/Em = 535/587 nm) was measured kinetically for 10–40 min. For the calculation, we first selected two points (T1 and T2) in the linear range of the plot and acquired the corresponding fluorescence (RFU1 and RFU2), then calculated the slope (MAO-B activity) for all samples by dividing the net ΔRFU values (RFU2-RFU1) by the time ΔT (T2-T1).

2.6. Reversibility test

The recovery of MAO-B enzyme activity was analyzed as reported with modifications (Park et al., 2019). Briefly, MAO-B (80 μl; final concentration, 300 nM) were incubated with DMB (80 μl; final concentration, 30 μM), safinamide (final concentration, 50 nM) or selegiline (final concentration, 50 nM) for 30 min and then divided into two aliquots. One aliquot (10 μl) was incubated with 5 μl of [Amplex Red reagent (final concentration, 200 μM), HRP (final concentration, 1 U/ml)] and 5 μl of tyramine hydrochloride (final concentration, 400 μM). The mixture was incubated for 60 min at room temperature and then the MAO-B enzyme activity was tested. Another aliquot (100 μl) was used for the washout experiment using Slide-A-Lyzer MINI Dialysis Device. 24 h after dialysis, 10 μl of solution was collected for measurement of the remaining enzyme activity. Safinamide (reversible MAO-B inhibitor) and selegiline (irreversible inhibitor) were used as positive control.

2.7. Molecular docking analysis

Molecular docking analysis was carried out using Molecular Operating Environment (MOE, version 2019.01, Chemical Computing Group Inc., Montreal, QC, Canada). The molecular model of MAO-B was built from the X-ray co-crystal structure of the human MAO-B in complex with the selective inhibitor safinamide (PDB ID: 2V5Z) according to the literature (Binda et al., 2007). Docking experiments were carried out using the default parameters of MOE as previously reported method (Guo et al., 2017).

2.8. Statistical analysis

For the biological investigation, data were acquired from three independent experiments. Data analysis was performed using SPSS software 25.0. One-way ANOVA followed by LSD Post Hoc Test was used for statistical comparisons and $p < 0.05$ or less was taken as statistically significant.

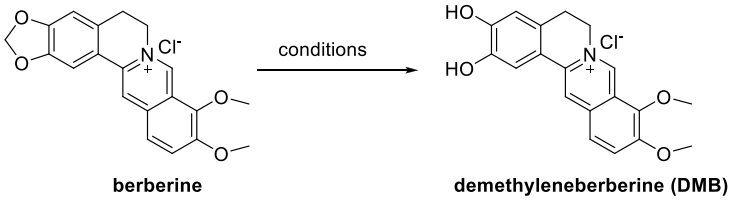
3. Results

3.1. Chemical synthesis of DMB, columbamine and palmatine

We envisaged that the main challenge for the semi-synthesis strategy of berberine alkaloids would arise from the specificity of the generating quaternary ammonium salts. The difference of solubility and polarity between quaternary ammonium salts and other organic compounds makes it hard to isolate the products by using normal synthetic methods. Also, the harsh conditions always led to the decomposition of products. These vexing problems could be addressed by finding suitable isolating methods and mild reaction conditions to avoid decomposition. With this idea in mind, we started our synthesis.

As for the pivotal demethylation reaction (Table 1), a variety of

Table 1
Synthesis of DMB.

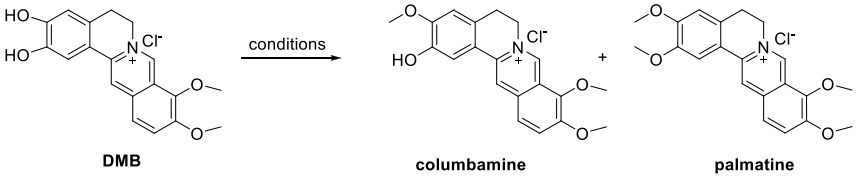


entry	Conditions	temp (°C)	time (h)	yield (%)
1	60% H_2SO_4, phloroglucinol	80	0.2	10
1	60% H_2SO_4, phloroglucinol	80	0.4	5
2	conc. HCl, water	25–100	36	0
3	conc. HCl, phloroglucinol	25–100	36	0
4	conc. HCl	25–100	36	0
5	conc. HCl, TsOH	25–100	36	0
6	60% H_2SO_4, TsOH	25–100	36	0
7	$AlCl_3$, TsOH, xylene	25–100	36	0
8	TsOH, xylene	25–100	36	0
9	TFA, xylene	25–100	36	0
10	**TfOH, xylene then 1 M HCl**	**25**	**0.5**	**90**
11	TfOH, xylene then 1 M HCl	25	1	70

inorganic acid conditions were tested, but all of them turn out to be fruitless, leading to no reaction or decomposition under more harsh conditions (entries 1–6). Then we turned our attention to examine the organic acid (entries 7–11). Much to our delight, after numerous unsuccessful trials, we found that treatment of berberine with trifluoromethanesulfonic acid (TfOH) in the presence of xylene at room temperature successfully delivered the desired demethylation product DMB. During the reaction condition screening, we found that 30 min was the optimal reaction time (entry 10) and expanding the reaction time led to decomposition (entry 11). Furthermore, 1 M HCl was found crucial for the precipitation of DMB. It is noteworthy that our present method turned out to be considerable practical: the desired natural product DMB was produced in 90% yield on a multigram scale with simple purification operation by utilizing low-cost and environmentally-friendly reagents.

With the multigram-scale access to DMB realized, we next turned our attention to further improve the efficacy for the methylation protocol and the completing the synthesis of columbamine and palmatine (Table 2). Many standard methylation conditions including MeI and dimethyl sulfate (Me_2SO_4) were tested. We found that DMB wasn't completely consumed under the conditions of MeI (1.2–2.5 equiv) or Me_2SO_4 (1.2–2.5 equiv) (entries 1, 2, 4, 5), and further optimizations including increasing the reagent equivalent or temperature led to the major double-methylation product palmatine (entries 3, 6). In contrast, the inertial and low-toxicity methylation reagent methyl *p*-toluenesulfonate (TsOMe) was then investigated (entries 7–12). To our delight, TsOMe/K_2CO_3 system successfully delivered the desired monomethylation product columbamine by precisely controlling reagent equivalent, dropping rate, temperature and reaction time (entry 10). Furthermore, increasing the reagent equivalent also delivered the double-methylation product palmatine in an efficient and eco-friendly way (entry 12).

Table 2
Synthesis of columbamine and palmatine.



entry	methylation reagent (equiv)	base (equiv)	solvent[a]	temp (°C)	time (h)	results
1	MeI (1.2)	NaOH (1.1)	MeOH	25	12	incomplete conversion[b]
2	MeI (2.5)	NaOH (1.1)	MeOH	25	36	incomplete conversion[b]
3	MeI (4)	NaOH (2.5)	MeOH	25	6	palmatine (60% yield)
4	Me_2SO_4 (1.2)	K_2CO_3 (1.1)	MeOH	60	12	incomplete conversion[b]
5	Me_2SO_4 (2.5)	K_2CO_3 (1.1)	MeOH	60	36	incomplete conversion[b]
6	Me_2SO_4 (4)	K_2CO_3 (2.5)	MeOH	60	6	palmatine (75% yield)
7	TsOMe (1.1)	K_2CO_3 (1.0)	MeCN	25	24	no reaction
8	TsOMe (1.1)	K_2CO_3 (1.0)	MeCN	40	6	incomplete conversion[b]
9	TsOMe (1.1)	K_2CO_3 (1.0)	MeCN	60	4	incomplete conversion[b]
10	**TsOMe (1.1)**	**K_2CO_3 (1.0)**	**MeCN**	**60**	**6**	**columbamine (60%)**
11	TsOMe (1.1)	K_2CO_3 (1.0)	MeCN	60	8	columbamine (30% yield) palmatine (20% yield)
12	**TsOMe (3)**	**K_2CO_3 (2.2)**	**MeCN**	**80**	**12**	**palmatine (80% yield)**

[a] Concentration: 0.027 M and concentration variation failed to give better results (entries 1–11); 0.1 M (entry 12).
[b] A large amount of DMB was remained.

3.2. DMB inhibited MAO-B with higher potency and efficacy than columbamine

We first tested the inhibitory effects of DMB and columbamine on MAO-B activity using a commercial kit. It was evident in Fig. 3 that DMB almost fully inhibited MAO-B with an IC_{50} value of 9.06 μM, and columbamine decreased the enzyme activity to around 60% of the control value at 100 μM.

3.3. DMB reversibly inhibited MAO-B enzyme activity

We further confirmed the reversibility of DMB on MAO-B enzyme activity in a wash-and-recover experiment. As indicated in Fig. 4, DMB and safinamide (a known reversible MAO-B inhibitor) reversibly inhibited MAO-B because catalytic activity of this enzyme was recovered by dialysis, with the activities at 93.8 ± 1.8% and 59.8 ± 2.9%, compared to the control. For comparison, MAO-B inhibition by DMB and safinamide persisted in undialyzed samples with the activities at 27.5 ± 1.6% and 42.4 ± 1.3%, respectively. However, dialysis failed to restore catalytic activity when MAO-B was incubated with selegiline.

3.4. DMB interacted with MAO-B via the formation of hydrogen bond

Furthermore, in order to clarify the binding mode of DMB and columbamine with the active sites of MAO-B, docking simulation studies of these compounds were performed by MOE software. As demonstrated in Fig. 5, DMB and columbamine were completely buried inside the pocket of MAO-B, a mechanism similar to the reversible inhibitor safinamide. Specifically, DMB formed hydrogen bond to Ile199 and arene-hydrogen bond to Cys172, respectively (Fig. 5B). In comparison, columbamine formed arene-arene and arene-hydrogen bonds to Tyr398 and Tyr326 in MAO-B, respectively (Fig. 5C).

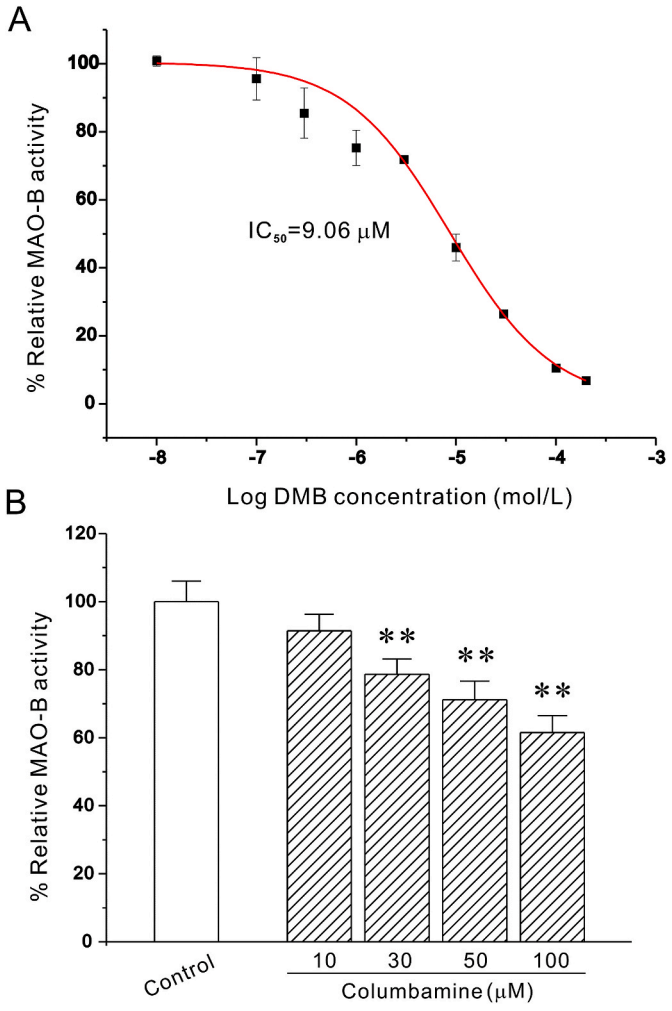


Fig. 3. DMB inhibited MAO-B enzyme activity more potently and more efficaciously than columbamine. DMB or columbamine was incubated with MAO-B enzyme in 96-well black plates for 30 min at 37 °C, then the solution of MAO-B substrate mixture was added into the well. The fluorescence was measured (Ex/Em = 535/587 nm) kinetically for 10–40 min at 37 °C.

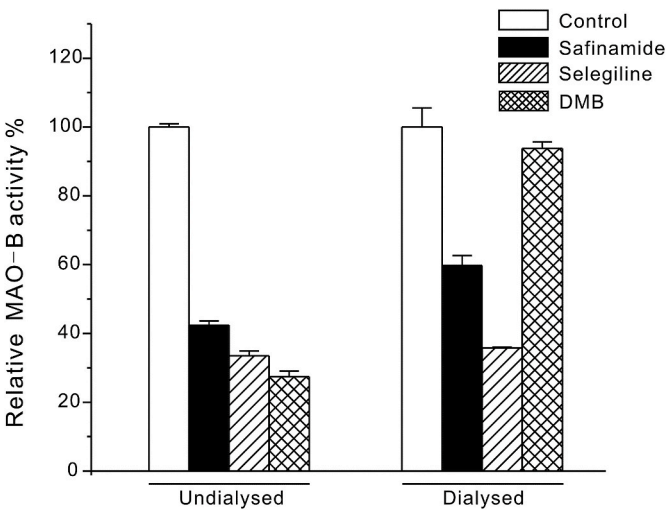


Fig. 4. DMB reversibly inactivated MAO-B. MAO-B was incubated with DMB, selegiline or safinamide for 30 min and dialyzed for 24 h, and the enzyme activity was determined.

4. Discussion

As such, the vexing bioinspired semi-synthesis problem has been elegantly solved. Literature precedents of the demethylation reaction are mainly confined to the use of sulfuric acid (Cava and Reed, 1967; Li et al., 2010; Spinozzi et al., 2014). This method turned out to be rather inefficient, because the harsh condition (60% H_2SO_4, 95 °C) resulted in large amount of by-products, which led to the low yield of final product DMB (40% yield). Also, the complex purification operation impeded the large-scale production of DMB. In contrast, our method solved the above problems: we found that the TfOH/xylene method delivered the desired demethylation product DMB in 90% yield with little by-products. Furthermore, the mild condition (i.e., room temperature, high reaction concentration) coupled with simple reaction operation make this reaction to be carried out easily in multi-gram scale. In addition, we provided an eco-friendly way for the synthesis of columbamine and palmatine by utilizing less toxic methylation reagent. Also, it should be noted that the TsOMe/K_2CO_3 method was found to have a better selectivity for the mono-methylation due to the relative inertial reactivity of TsOMe, whereas other standard methylation conditions, such as MeI and Me_2SO_4 always led to incomplete conversion in the same condition or large amount of double-methylation product when increasing the reagent equivalent. The combination of all these features resulted in exceptional synthetic efficiency of our methods.

The interest of scientists in the discovery of MAO-B inhibitors is rising steadily due to the therapeutic significance of these inhibitors in PD treatment. It has been previously reported that among the berberine analogues, jatrorrhizine was able to inactivate MAO-B with an IC_{50} value of 62 μM. While berberine and palmatine exhibited no inhibition (Kong et al., 2001). So far, due to the shortage in the natural supply and difficulty in chemical synthesis, the anti-MAO-B capacity of other berberine derivatives, DMB and columbamine, remained unknown. In the current study, we found that DMB was able to almost completely inhibit MAO-B enzyme activity with an IC_{50} value of 9.06 μM.

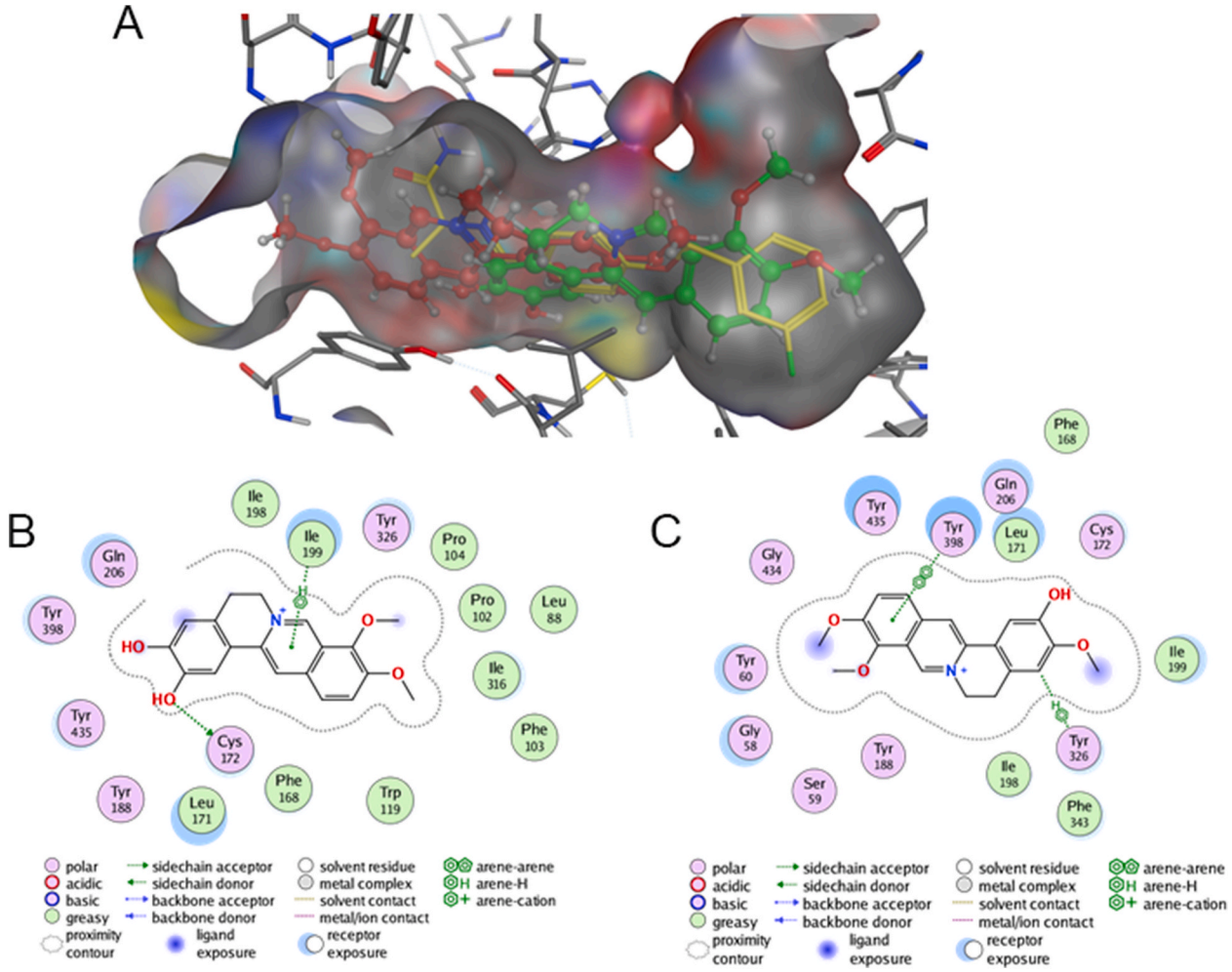


Fig. 5. The proposed binding mode of DMB and columbamine with MAO-B. (A), The compounds DMB (green) and columbamine (red) bond in the pocket of MAO-B (PDB ID: 2V5D) in a similar pose with the ligand safinamide (yellow) of the co-crystal structure. 2D ligand interaction depiction of the docked compounds DMB (B) and columbamine (C) within the pocket of MAO-B.

Columbamine showed a moderate inhibitory effect by decreasing enzyme activity to 60% of the control value. These findings indicated that the order of MAO-B inhibitory activity was: DMB > jatrorrhizine > columbamine > berberine = palmatine.

From a structural perspective, all of these five natural products share the same protoberberine molecular skeleton with only slight substitution differences. The difference of their MAO-B inhibitory effects may correlate with the substituent groups at C2 and C3 positions. Specifically, berberine and palmatine without hydroxyl group showed no inhibition on MAO-B. In contrast, jatrorrhizine and columbamine with one hydroxyl group exhibited mild MAO-B inhibitory activity and DMB with two hydroxyl groups demonstrated the highest MAO-B inhibitory activity. These observations indicate that the hydrophilic hydroxyl groups at C2 and C3 positions are likely to enhance the MAO-B inhibitory activity, while the methoxy and methylenedioxy groups at these positions have an opposite effect. In addition,a comparison of jatrorrhizine against columbamine indicates that C3-hydroxyl group is essential for the MAO-B inhibitory activity. This result may be due to the high reactivity of C3-hydroxyl group. Owing to the conjugate effect with quinonoid, C3-hydroxyl group exhibits higher acidity than C2-hydroxyl group.

On the other hand, as demonstrated by earlier studies (Binda et al., 2003; Milczek et al., 2011), the residue of Ile199 plays critical roles in connecting the entrance cavity to the substrate cavity, and hence it is one of the important residue for MAO-B selectivity. Our findings that DMB (but not columbamine) generated electrostatic interaction with Ile199 may also explain why DMB displayed MAO-B inhibitory effects more potently and efficaciously than columbamine.

Furthermore, DMB and jatrorrhizine were demonstrated herein and previously to be reversible MAO-B inhibitors (Kong et al., 2001), a conclusion supported by the fact that they caused a huge recovery of enzyme activity after dialysis. In comparison, selegiline and rasagiline are two irreversible MAO-B antagonists used clinically to treat PD with an IC_{50} value of 6.8–40 nM and 14–66 nM, respectively (Can et al., 2017; Youdim et al., 2001). Generally speaking, these irreversible inhibitors inactivate MAO-B enzyme more potently than reversible ones due to the covalent mechanism. However, reversible MAO-B inhibitors, particular those from natural herbs, possess substantial merit in terms of safety and side effect profile. As demonstrated by an earlier study (Park et al., 2019), irreversible inhibitors covalently modify MAO-B enzyme and will finally cause permanent damage to the enzyme itself. As the turnover rate of the MAO-B enzyme in the brain is quite slow, this injury would recruit other enzymes to compensate for MAO-B deficiency. In contrast, reversible inhibitors occupy MAO-B enzyme active site and compete with substrates, resulting in an intact MAO-B enzyme. More notably, in addition to MAO-B inhibitory effects, DMB also exhibits a variety of pharmacological activities against neurological diseases, including anti-oxidation (Qiang et al., 2016) and anti-inflammation (Zhang et al., 2020) and anti-mitochondrial dysfunction (Zhang et al., 2015). These characteristics may enable DMB to effectively combat PD by hitting the multiple targets in the brain concurrently and simultaneously. On the other hand, we have addressed the large-scale synthetic problem of DMB, which guarantees sufficient quantities of DMB for further structural derivation and biological evaluation. We presume this would be another advantage of DMB compared with other high-cost drugs.

In summary, we have elegantly solved the vexing bioinspired semi-synthesis problem of berberine alkaloids, by showcasing a remarkable progress in the reaction yield, production scale, reagent toxicity compared with previous work. Biologically, we have provided solid evidence that berberine analogues, DMB in particular, could reversibly inhibit MAO-B enzyme activity and the inhibitory effects were achieved possibly through the hydrogen bond to Ile199 and arene-hydrogen bond to Cys172. Our work would encourage further pharmacological studies of berberine alkaloids and provide valuable insight for the semi-synthesis of berberine alkaloids.

Author statement

Cheng Tao: Conceptualization, Methodology, Investigation, Writing - Original Draft. **Sheng-quan Hu**: Conceptualization, Methodology, Investigation, Writing - Original Draft. **Jian Chen**: Resources, Formal analysis, Data Curation. **Yuan-ji Chen**: Data Curation. **Ke-huan Sun**: Funding acquisition. **Guo-zhen Cui**: Methodology, Software, Formal analysis. **Min Ma**: Project administration. **Zheng-zhi Wu**: Supervision, Funding acquisition, Project administration.

Acknowledgements

This study was supported by Natural Science Foundation of China (No. 81574038), Shenzhen Basic Discipline Layout Project (No. JCYJ20170412161254416, JCYJ20170413161352000 and JCYJ20170306170933370), the Shenzhen Municipal Science & Technology Project (JCYJ20170818094033689, JCYJ20190812171005713), Guangdong Basic and Applied Basic Research Foundation (2019A1515110874), Natural Science Foundation of Guangdong Province (NO. 2018A0303130158), National High Technology Research and Development Program (No. 2014AA020907), Shenzhen Sanming Project of Medical and Health (No. SZSM 201612049), Guangdong Science and Technology Department Public Welfare Research Project (No. 2015B020211001), the Science and Technology Foundation of Guangdong Province of China (No. 2017A050506007).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.neuint.2020.104807.

References

Binda, C., Li, M., Hubalek, F., Restelli, N., Edmondson, D.E., Mattevi, A., 2003. Insights into the mode of inhibition of human mitochondrial monoamine oxidase B from high-resolution crystal structures. Proc. Natl. Acad. Sci. U. S. A. 100, 9750–9755.

Binda, C., Wang, J., Pisani, L., Caccia, C., Carotti, A., Salvati, P., Edmondson, D.E., Mattevi, A., 2007. Structures of human monoamine oxidase B complexes with selective noncovalent inhibitors: safinamide and coumarin analogs. J. Med. Chem. 50, 5848–5852.

Can, O.D., Osmaniye, D., Demir Ozkay, U., Saglik, B.N., Levent, S., Ilgin, S., Baysal, M., Ozkay, Y., Kaplancikli, Z.A., 2017. MAO enzymes inhibitory activity of new benzimidazole derivatives including hydrazone and propargyl side chains. Eur. J. Med. Chem. 131, 92–106.

Carradori, S., D'Ascenzio, M., Chimenti, P., Secci, D., Bolasco, A., 2014. Selective MAO-B inhibitors: a lesson from natural products. Mol. Divers. 18, 219–243.

Castillo, J., Hung, J., Rodriguez, M., Bastidas, E., Laboren, I., Jaimes, A., 2005. LED fluorescence spectroscopy for direct determination of monoamine oxidase B inactivation. Anal. Biochem. 343, 293–298.

Cava, M.P., Reed, T.A., 1967. A synthesis of columbamine from berberine. J. Org. Chem. 32, 1640–1641.

Chen, Y., Chen, Y., Liang, Y., Chen, H., Ji, X., Huang, M., 2020. Berberine mitigates cognitive decline in an Alzheimer's Disease Mouse Model by targeting both tau hyperphosphorylation and autophagic clearance. Biomed. Pharmacother. 121, 109670.

Guo, B., Hu, S., Zheng, C., Wang, H., Luo, F., Li, H., Cui, W., Yang, X., Cui, G., Mak, S., Choi, T.C., Ma, E.D., Wang, Y., Lee, S.M.Y., Zhang, Z., Han, Y., 2017. Substantial protection against MPTP-associated Parkinson's neurotoxicity in vitro and in vivo by anti-cancer agent SU4312 via activation of MEF2D and inhibition of MAO-B. Neuropharmacology 126, 12–24.

Hanaoka, M., Hirasawa, T., Cho, W.J., Yasuda, S., 2000. Convenient synthesis of 2,3,9,10-tetraoxygenated protoberberine alkaloids and their 13-methyl alkaloids. Chem. Pharm. Bull. (Tokyo) 48, 399–404.

Kim, D.G., Choi, J.W., Jo, I.J., Kim, M.J., Lee, H.S., Hong, S.H., Song, H.J., Bae, G.S., Park, S.J., 2019. Berberine ameliorates lipopolysaccharideinduced inflammatory responses in mouse inner medullary collecting duct3 cells by downregulation of NFkappaB pathway. Mol. Med. Rep. 21, 258–266.

Kong, L.D., Cheng, C.H., Tan, R.X., 2001. Monoamine oxidase inhibitors from rhizoma of Coptis chinensis. Planta Med. 67, 74–76.

Li, Y.H., Li, Y., Yang, P., Kong, W.J., You, X.F., Ren, G., Deng, H.B., Wang, Y.M., Wang, Y. X., Jiang, J.D., Song, D.Q., 2010. Design, synthesis, and cholesterol-lowering efficacy for prodrugs of berberrubine. Bioorg. Med. Chem. 18, 6422–6428.

Milczek, E.M., Binda, C., Rovida, S., Mattevi, A., Edmondson, D.E., 2011. The 'gating' residues Ile199 and Tyr326 in human monoamine oxidase B function in substrate and inhibitor recognition. FEBS J. 278, 4860–4869.

Orejarena Pacheco, J.C., Lahm, G., Opatz, T., 2013. Synthesis of alkaloids by Stevens rearrangement of nitrile-stabilized ammonium ylides: (+/-)-laudanosine, (+/-)-laudanidine, (+/-)-armepavine, (+/-)-7-methoxycryptopleurine, and (+/-)-xylopinine. J. Org. Chem. 78, 4985–4992.

Orito, K., Miyazawa, M., Kanbayashi, R., Tokuda, M., Suginome, H., 1999. Synthesis of phthalideisoquinoline and protoberberine alkaloids and indolo[2,1-a]isoquinolines in a divergent route involving palladium(0)-catalyzed carbonylation(1). J. Org. Chem. 64, 6583–6596.

Park, J.H., Ju, Y.H., Choi, J.W., Song, H.J., Jang, B.K., Woo, J., Chun, H., Kim, H.J., Shin, S.J., Yarishkin, O., Jo, S., Park, M., Yeon, S.K., Kim, S., Kim, J., Nam, M.H., Londhe, A.M., Kim, J., Cho, S.J., Cho, S., Lee, C., Hwang, S.Y., Kim, S.W., Oh, S.J., Cho, J., Pae, A.N., Lee, C.J., Park, K.D., 2019. Newly developed reversible MAO-B inhibitor circumvents the shortcomings of irreversible inhibitors in Alzheimer's disease. Sci Adv 5, eaav0316.

Qiang, X., Xu, L., Zhang, M., Zhang, P., Wang, Y., Wang, Y., Zhao, Z., Chen, H., Liu, X., Zhang, Y., 2016. Demethyleneberberine attenuates non-alcoholic fatty liver disease with activation of AMPK and inhibition of oxidative stress. Biochem. Biophys. Res. Commun. 472, 603–609.

Reddy, V., Jadhav, A.S., Vijaya Anand, R., 2015. A room-temperature protocol to access isoquinolines through Ag(I) catalysed annulation of o-(1-alkynyl)arylaldehydes and ketones with NH4OAc: elaboration to berberine and palmatine. Org. Biomol. Chem. 13, 3732–3741.

Rueffer, M., Zenk, M.H., 1986. Columbamine, the central intermediate in the late stages of protoberberine biosynthesis. Tetrahedron Lett. 27, 923–924.

Spinozzi, S., Colliva, C., Camborata, C., Roberti, M., Ianni, C., Neri, F., Calvarese, C., Lisotti, A., Mazzella, G., Roda, A., 2014. Berberine and its metabolites: relationship between physicochemical properties and plasma levels after administration to human subjects. J. Nat. Prod. 77, 766–772.

Tao, D., Wang, Y., Bao, X.Q., Yang, B.B., Gao, F., Wang, L., Zhang, D., Li, L., 2019. Discovery of coumarin Mannich base derivatives as multifunctional agents against monoamine oxidase B and neuroinflammation for the treatment of Parkinson's disease. Eur. J. Med. Chem. 173, 203–212.

Tarabasz, D., Kukula-Koch, W., 2019. Palmatine: a review of pharmacological properties and pharmacokinetics. Phytother Res. 34, 33–50.

Tong, S., Yaan, J., Lou, J., 2005. Preparative isolation and purification of alkaloids from Corydalis yanhusuo W. T. Wang by high speed counter-current chromatography. J. Liq. Chromatogr. Relat. Technol. 28, 2979.

Tzvetkov, N.T., Stammler, H.G., Neumann, B., Hristova, S., Antonov, L., Gastreich, M., 2017. Crystal structures, binding interactions, and ADME evaluation of brain penetrant N-substituted indazole-5-carboxamides as subnanomolar, selective monoamine oxidase B and dual MAO-A/B inhibitors. Eur. J. Med. Chem. 127, 470–492.

Wang, L.H., Li, X.L., Li, Q., Fu, Y., Yu, H.J., Sun, Y.Q., Zhang, L., Shan, H.L., 2012a. Berberine alleviates ischemic arrhythmias via recovering depressed I(to) and I(Ca) currents in diabetic rats. Phytomedicine 19, 206–210.

Wang, Y.X., Kong, W.J., Li, Y.H., Tang, S., Li, Z., Li, Y.B., Shan, Y.Q., Bi, C.W., Jiang, J.D., Song, D.Q., 2012b. Synthesis and structure-activity relationship of berberine analogues in LDLR up-regulation and AMPK activation. Bioorg. Med. Chem. 20, 6552–6558.

Wang, J., Jiang, Y., Wang, B., Zhang, N., 2019. A review on analytical methods for natural berberine alkaloids. J. Separ. Sci. 42, 1794–1815.

Youdim, M.B., Gross, A., Finberg, J.P., 2001. Rasagiline [N-propargyl-1R(+)-aminoindan], a selective and potent inhibitor of mitochondrial monoamine oxidase B. Br. J. Pharmacol. 132, 500–506.

Yu, Q., Tong, S., Yan, J., Hong, C., Zhai, W., Li, Y., 2011. Preparative separation of quaternary ammonium alkaloids from Corydalis yanhusuo W. T. Wang by pH-zone-refining counter-current chromatography. J. Separ. Sci. 34, 278–285.

Zhang, P., Qiang, X., Zhang, M., Ma, D., Zhao, Z., Zhou, C., Liu, X., Li, R., Chen, H., Zhang, Y., 2015. Demethyleneberberine, a natural mitochondria-targeted antioxidant, inhibits mitochondrial dysfunction, oxidative stress, and steatosis in alcoholic liver disease mouse model. J. Pharmacol. Exp. Therapeut. 352, 139–147.

Zhang, M., Li, Q., Zhou, C., Zhao, Y., Li, R., Zhang, Y., 2020. Demethyleneberberine attenuates concanavalin A-induced autoimmune hepatitis in mice through inhibition of NF-kappaB and MAPK signaling. Int. Immunopharm. 80, 106137.