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# The Natural Product Berberine is a Human Prolyl Oligopeptidase Inhibitor

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*Prolyl oligopeptidase is a cytosolic serine peptidase that hydrolyzes proline-containing peptides at the carboxy terminus. This peptidase has been associated with schizophrenia, bipolar affective disorder, and related neuropsychiatric disorders, and therefore may have important clinical implications. Among the strategies used to find novel prolyl oligopeptidase inhibitors, traditional Chinese medicinal plants provide a rich source of unexplored compounds. We used $^{19}F$ NMR spectroscopy to search for new prolyl oligopeptidase inhibitors in a library of traditional Chinese medicine plant extracts. Several extracts were identified as powerful inhibitors of this peptidase. The alkaloid berberine was the prolyl oligopeptidase inhibitory molecule isolated from* Rhizoma coptidis *extract. Berberine inhibited prolyl oligopeptidase in a dose-dependent manner. As berberine is a natural compound that has been safely administered to humans, it opens up new perspectives for the treatment of neuropsychiatric diseases. The results described herein suggest that the initiation of clinical trials in patients with schizophrenia, bipolar affective disorder, or related diseases in which cognitive capabilities are affected should be undertaken with either the extract or pure BBR.*

## Introduction

Schizophrenia (SZ) is a serious mental illness that affects approximately 1% of the world's population.[1] Treatment of this disorder constitutes one of the main items in most health budgets in developed countries; in the United States the costs of SZ have been estimated to account for 2.5% of the total health budget whereas in Europe the figure is 1.9%.[2] The symptoms presented in SZ can be classified into three categories: positive (for example, auditory and visual hallucinations), negative (for example, decreased social interaction), and cognitive deficits (for example, impairments in executive function, working memory, and attention).[3] Antipsychotic drugs are the main agents used to treat SZ; however, 30% of patients continue to have severe and persistent symptoms even when receiving effective doses of these medications, and more than 60% have some residual symptoms despite adequate treatment.[4] Whereas positive and negative symptoms have long been considered the hallmark of SZ, recent clinical studies have highlighted cognitive dysfunction as a third major diagnostic category which is increasingly considered to be the core deficit of the disorder. SZ has an enormous economic impact, as evidenced by the fact that up to 90% of patients are unemployed. This impact is largely attributable to the lack of adequate therapeutic agents targeted to treat the key cognitive symptoms. Accordingly, a new class of drugs is required that addresses the cognitive deficits in this disease.[5] Treatment regimes designed to improve psychotic and cognitive symptoms must include the administration of traditional antipsychotic medications combined with cognition enhancement treatments.[6]

Another prevalent mental illness with high social impact is bipolar affective disorder (BD). The mood-stabilizing drugs commonly used to treat this disorder are often highly effective in controlling symptoms but in many cases intolerable side-effects make them unacceptable.[7]

In recent years, the protease prolyl oligopeptidase (POP; EC 3.4.21.26) has gained importance as a target for treatment of the above diseases, particularly because of its involvement in the metabolism of inositol-1,4,5-$P_3$ ($IP_3$). $IP_3$ is a key molecule in the transduction cascade of neuropeptide signaling. Neuropeptides induce and increase $IP_3$ levels, which binds to its receptor in the membrane of endoplasmatic reticulum and induces the release of $Ca^{2+}$, which is believed to play a crucial role in learning and memory.[8] Recent findings have demonstrated that POP modulates the concentration of $IP_3$. Disruption of the POP gene in the lower eukaryote *Dictyostelium discoideum* induces lithium resistance by elevation of $IP_3$.[9] Also the reduced proteolytic activity of POP is responsible for elevated $IP_3$ concentration in POP antisense human glioma cells. The same effect is observed when these cells are treated with specific POP inhibitors.[10] Moreover, the mood-stabilizing drug commonly used to treat BD, valproic acid, directly inhibits recombinant POP activity.[7] The $IP_3$ signaling pathway is involved in the therapeutic action of several mood-stabilizing drugs (lith-

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ium, carbamazepine, and valproic acid)[11] and defects in the mechanisms that regulate $IP_3$ signaling may underlie BD. This observation also indicates that other small molecule inhibitors of POP may be useful in the treatment of BD.[7]

In clinical studies, patients with BD or SZ exhibit increased levels of serum POP activity.[12] POP inhibitors might be valuable compounds in a variety of clinical conditions of the brain, as indicated by the neuroprotective and cognition-enhancing effects of POP inhibitors in experimental animals.[13–15] In addition, the POP inhibitor S-17092-1 has been tested in Phase I trials for its capacity to enhance cognition.[16]

POP is a cytosolic serine protease that hydrolyses proline-containing peptides at the carboxy terminus of proline-residues.[17,18] In our laboratory, POP was recently cloned from human brain RNA, expressed in *E. coli* and an homology model based on the X-ray structure of porcine POP was obtained.[19]

In spite of promising results, a drug based on POP inhibition has yet to reach the market. A range of strategies are currently being used to identify POP inhibitors, among these, natural products are one of the main sources of new inhibitory agents.[20–22] Traditional Chinese Medicine (TCM) dates back several thousands of years. China registers 12,806 medicinal resources, including 11,145 plants.[23] Moreover, 2,375 compounds are compiled in the Pharmacopoeia of the Peoples Republic of China (2000 edition).[24] Compounds with anticancer, antibacterial, antifungal, and antiviral activities have been identified from these medicinal products.[25] Such a large number of medicinal plants provides a rich pool from which to discover new drugs of natural origin.

We recently reported a new enzymatic assay based on the combined use of $^{19}F$ NMR spectroscopy and the fluorinated substrate ZGPF-4-$CF_3$. This novel assay is fast, reproducible, and circumvents the false positive and false negative problems of the fluorimetric and colorimetric assays previously reported. This assay may therefore be useful for screening complex natural compound mixtures for new POP inhibitors.[26]

We applied the $^{19}F$ NMR spectroscopy based POP inhibitory assay previously reported to screen a large library of plant extracts used in TCM for POP inhibitory properties. Several extracts showed potent inhibitory action of this peptidase, and the alkaloid berberine (BBR) was purified and identified as one of the POP inhibitory compounds present.

## Results

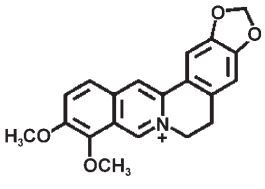
We prepared a library of aqueous extracts from 29 plants used in TCM and screened them for their capacity to inhibit POP. Specifically, we focused on plants used to treat neuropsychiatric disorders.[27] To monitor the POP inhibitory activity of the extracts, we used a previously reported $^{19}F$ NMR POP inhibition assay.[26] The assay is based on the use of the fluorine-labeled POP substrate, Z-Gly-Pro-Phe-4($CF_3$)-$NH_2$ (ZGPF-4-$CF_3$), which allows the monitoring of POP inhibitory activity by means of $^{19}F$ NMR spectroscopy and circumvents the false positive or false negative problems associated with commonly used fluorimetric and colorimetric assays. Several plant extracts, such as TCM 3, TCM 14, TCM 20, and TCM 40, showed potent POP inhibitory activity. Moreover, TCM 6, TCM 9, TCM 16, TCM 19, TCM 25, and TCM 33 completely inhibited POP activity (Table 1).

**Table 1.**
Percentages of POP inhibition shown by TCM extracts.

| Name | Abbreviation | Percentage inhibition[a] |
|---|---|---|
| *Herba hedyotis diffusae* | TCM 3 | 76 |
| *Radix Paeoniae rubra* | TCM 6 | 100 |
| *Arillus longan* | TCM 8 | 40 |
| *Cortex cinnamoni* | TCM 9 | 100 |
| *Rhizoma chuanxiong* | TCM 11 | 52 |
| *Radix ginseng* | TCM 12 | 12 |
| *Herba dendrobii* | TCM 14 | 79 |
| *Rhizoma polygonati odorati* | TCM 15 | 68 |
| *Rhizoma coptidis* | TCM 16 | 100 |
| *Carapax trioncys* | TCM 17 | 0 |
| *Poria* | TCM 18 | 73 |
| *Radix scutellariae* | TCM 19 | 100 |
| *Fructus gardeniae* | TCM 20 | 53 |
| *Colla corii asini* | TCM 22 | 0 |
| *Ganoderma* | TCM 23 | 36 |
| *Radix rehmanniae* | TCM 24 | 13 |
| *Flos inulae* | TCM 25 | 58 |
| *Radix angelicae sinensis* | TCM 30 | 25 |
| *Radix curcumae* | TCM 32 | 27 |
| *Ramullus cinnamoni* | TCM 33 | 100 |
| *Radix notoginseng* | TCM 38 | 0 |
| *Herba thlaspis* | TCM 40 | 69 |
| *Fructus jujubae* | TCM 41 | 4 |
| *Cortex meliae* | TCM 42 | 74 |
| *Concretio silicea bambusae* | TCM 43 | 68 |
| *Fructus lycii* | TCM 44 | 17 |
| *Poria spirit* | TCM 45 | 78 |
| *Bombyx batryticatus* | TCM 46 | 32 |
| *Semen ziziphi spimosae* | TCM 47 | 39 |

[a] Measured using ZGPF-4-$CF_3$ as substrate. The final concentration of TCMs in the assay buffer was 100 $\mu g\,mL^{-1}$ (14 $\mu L$ of a TCM stock at 5 $mg\,mL^{-1}$ was added to the reaction mixture).

To continue our work, we focused our efforts on one of the extracts with greatest POP inhibitory capacity, TCM 16. The extract was fractionated by semipreparative HPLC and all the fractions were then collected. Subsequently, we evaluated the POP inhibitory activity of each fraction and identified those containing active molecules. After careful repurification of selected fractions by semipreparative HPLC, we selected one active fraction with a purity of more than 99%, as calculated from its HPLC profile (Figure 1). The retention time of the pure compound was 8.72 min in a gradient of MeCN from 15–65% during 15 min. The exact mass of the purified molecule was 336.1237 Da (Figure 1), which corresponded to the formula $C_{20}H_{18}NO_4$. Further characterization of the purified compound by $^1H$ and $^{13}C$ NMR spectroscopy allowed us to identify the active molecule as the alkaloid berberine (BBR). When we compared the purified compound with an authentic BBR sample the two products co-eluted when analyzed by HPLC



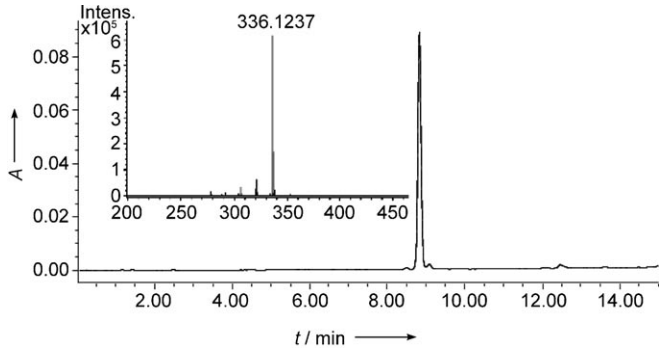



**Figure 1.** HPLC and exact mass MS spectra of purified BBR obtained from a *Rhizoma coptidis* aqueous extract.

(Figure 2), gave the same $^{1}H$ and $^{13}C$ spectra, and showed the same POP inhibitory activity. A $^{1}H$ NMR (800 MHz) showed unambiguously the presence of BBR diagnostic signals in the

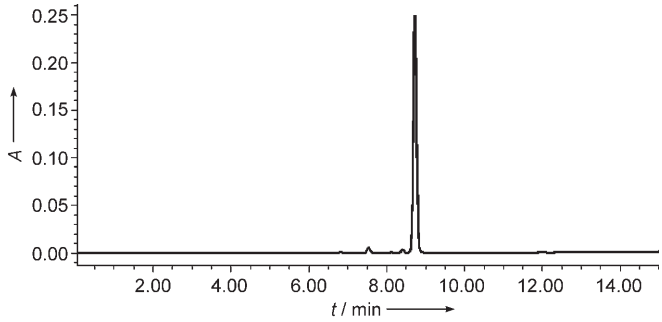



**Figure 2.** HPLC co-elution of BBR isolated from a *Rhizoma coptidis* aqueous extract and an authentic BBR sample.

whole *Rhizoma coptidis* extract (TCM 16) indicating that BBR was a component of the extract and has not been formed during the purification process (Figure 3). We constructed a calibration curve with known amounts of berberine to measure the amount of berberine in the extract. The extract of *Rhizoma coptidis* contained 23% berberine. On the basis of this data, we estimated that the amount of berberine in the inhibition assay performed with the plant extract was 68 μM. The amount of *Rhizoma coptidis* taken in TCM in one day is normally between 9 −12 g.[27] From this amount, and on the basis of our yields, 918–1224 mg of dry weight can be extracted when performing aqueous extracts. Given that the amount of berberine in the *Rhizoma coptidis* extract is 23%, we conclude that the dose of berberine taken in one day is 200–300 mg.

BBR inhibited POP in a dose-dependent manner (Figure 4). The concentration required for half-maximal inhibition ($IC_{50}$) was 145 ± 19 μM. The specificity of BBR against POP was studied using two proteases belonging to distinct families: HIV-1 protease, an aspartic protease, and bovine trypsin, a serine protease. BBR inhibited neither HIV-1 protease[28] nor trypsin at the same concentrations used to inhibit POP.

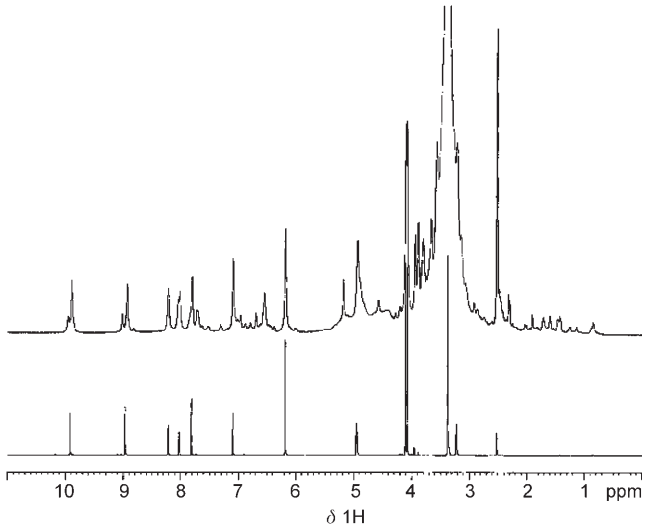



**Figure 3.** 800 MHz $^{1}H$ NMR of a *Rhizoma coptidis* extract (top) and an authentic berberine sample (bottom).

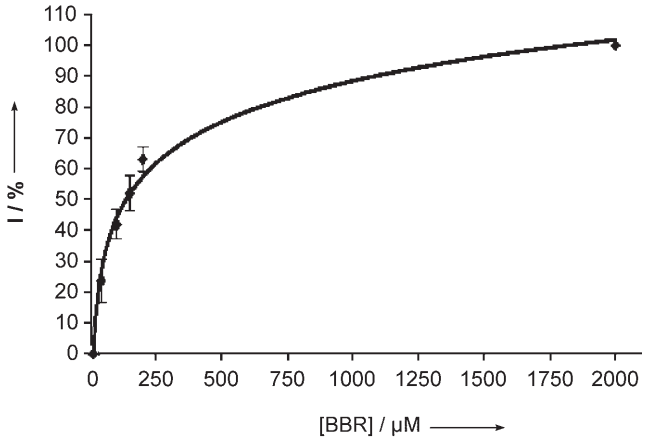



**Figure 4.** BBR $IC_{50}$ calculation curve using ZGP-AMC as substrate. Results are the mean ± standard deviations of three independent experiments.

## Discussion

The use of natural products for medical purposes is gaining international popularity. However, little is known about the mechanisms of action of their active principles.[29] After fractionation of the *Rhizoma coptidis* extract, we identified BBR as one of the molecules present that directly inhibited POP. To our knowledge, this is the first report of POP inhibitory activity of the alkaloid BBR. This molecule has a variety of pharmacological effects, including antidiarrheic[30] and antiarrhythmic activities.[31]

BBR is one of the main components of Huang Lian Jie Du Tang (Oren-gedoku-to), a traditional herbal prescription that protects against neuronal death induced by cerebral ischemia[32] and impairment of learning and memory induced by cerebral ischemia in mice.[33] However, previous studies did not elucidate the exact active components and the mechanism of action of Huang Lian Jie Du Tang for the treatment of brain

diseases.[34] Here we show that BBR directly inhibits POP in a dose-dependent manner, thus the effects observed when BBR is administered could be the result of POP inhibition. Nevertheless, as the $IC_{50}$ of berberine is 145 μM and the estimated amount of berberine in the inhibition assay performed with the plant extract is 68 μM, the extract must hold other POP inhibitory molecules. Accordingly, when we fractioned the extract, we found fractions that did not contain berberine but inhibited POP as well. These molecules are currently being characterized in our laboratory.

Furthermore, we also identified another component of the Huang Lian Jie Du Tang formulae, *Scutellariae radix*, as an extract with powerful POP inhibitory action. Work is now in progress in our laboratory to identify POP inhibitory molecules from this extract. A combination of several POP inhibitors in Huang Lian Jie Du Tang could explain the therapeutic effects reported, although the presence of other molecules that interact with distinct targets cannot be ruled out.

Recent findings have demonstrated that POP inhibition increases the concentration of $IP_3$,[9,10] a central molecule in the transduction cascade of neuropeptide signaling. $IP_3$ binds to its receptor in the membrane of endoplasmatic reticulum and induces the release of $Ca^{2+}$, which is believed to play a crucial role in learning and memory.[8] Moreover, neuroprotective and cognition-enhancing effects of POP inhibitors in experimental animals have been reported[13–15] and the POP inhibitor S-17092-1 has been tested in Phase I trials for its capacity to enhance cognition.[16] Recently, POP was identified as a binding partner of tubulin and was associated with the microtubulin cytoskeleton. Indeed, POP inhibition and POP antisense mRNA expression result in enhanced peptide/protein secretion from human U-343 glioma cells. These findings indicate that POP exerts novel functions in axonal transport and/or protein secretion; disturbance of these processes is associated with a number of ageing-associated neurodegenerative diseases.[35] Therefore, due to its POP inhibition effect, BBR may be of therapeutic use for many of the above-mentioned diseases.

Regarding the potency of berberine when compared with typical reference compounds such as S-17092-1,[16] it is well known that S-17092-1 is a covalent inhibitor that reacts with the serine in the enzyme active site. In contrast, berberine is probably a noncovalent inhibitor. It is logical that the potencies of a covalent and a noncovalent inhibitor differ by several orders of magnitude. From our point of view, the natural product berberine has other advantages, such as water solubility, absence of toxicity in humans, and capacity to reach the central nervous system (CNS). An important issue when developing a drug to target the CNS is its ability to cross the blood-brain barrier (BBB). In the case of BBR the experiments reported by Wang et al.[36] showed that after intravenous administration of *Rhizoma coptidis* extract, BBR crosses the BBB and reaches rat hippocampus. This observation indicates that BBR may have a direct effect on the neuron and may accumulate in the hippocampus. Another study showed that BBR is transported inside neurons in a concentration- and time-dependent manner.[37] All these data indicate that BBR will be able to reach the CNS, where POP is mainly located, without needing any chemical modification. These findings give added value to BBR as a drug that is able to reach the CNS.

Recently, two clinical trials involving BBR administration to humans have been reported. One addressed the efficacy and safety of BBR for chronic congestive heart failure and showed that the molecule improved quality of life and decreased mortality in these patients.[38] In the second, BBR was administered orally to 32 hypercholesterolemic patients for 3 months and proved to be a cholesterol-lowering drug.[39] In both trials, BBR was found to be a safe compound and no side effects were observed. The results described in the present paper demonstrating that BBR is able to inhibit POP suggest that the initiation of clinical trials in patients with SZ, BD, or related diseases in which cognitive capabilities are affected should be undertaken with either the extract or pure BBR.

## Conclusions

Here we demonstrated that the alkaloid BBR inhibits POP activity in a dose-dependent manner. As BBR is a natural product that has been already safely administered to humans, this compound opens up new perspectives for the treatment of SZ and BD. In particular, BBR may be of interest in therapies designed to improve the cognitive deficits of these patients.

## Experimental Section

Solvents for RP-HPLC were obtained from Scharlau (Barcelona, Spain). Trifluoroacetic acid was supplied from KaliChemie (Bad-Wimpfen, Germany). ZGP-AMC was obtained from Bachem (Bubendorf, Switzerland). Bovine Trypsin was purchased from Roche (Mannheim, Germany). Other chemicals, including a BBR sample, were purchased from Sigma–Aldrich (Deisenhofen, Germany).
HPLC was performed using a Waters Alliance 2695 (Waters, Massachusetts, USA) chromatography system with a PDA 995 detector, a reverse-phase Symmetry $C_{18}$ (4.6×150 mm) 5 μm column, using $H_2O$ with TFA (0.045%) and MeCN with TFA (0.036%) as mobile phases. Semipreparative HPLC was performed using a Waters Controller 600 chromatography system with a Fraction Collector II, Simple Manager 2700 autoinjector, and a 2478 UV/VIS detector. The column used was a Symmetry $C_{18}$ (30×100 mm) with a flow rate of 10 mL $min^{-1}$. The mobile phases used were $H_2O$ with TFA (0.1%) and MeCN with TFA (0.1%). Exact mass spectra were recorded on an ESI-TOF spectrometer (Bruker Microtof, Bremen, Germany). NMR spectra were recorded at 298 K on a Varian 400 NMR spectrometer in [$D_6$]DMSO and tetramethylsilane as internal standard. The $^1$H NMR spectra of pure BBR and TCM16 extract dissolved in [$D_6$]DMSO were recorded on a Bruker 800 MHz spectrometer at 298 K. Fluorescence was measured using a Bio-Tek FL600 fluorescence plate reader (Bio-Tek Instruments, Vermont, USA).

**Methods**

*TCM extract preparation*

Plants used in TCM were obtained from Herbasin (Shenyang, China). Dried plant material (30 g) was extracted with $H_2O$ (400 mL) under reflux for 5 h using a Soxhlet apparatus. The volume of aqueous extracts was reduced by evaporation under vacuum and afterwards freeze-dried and stored at −20 °C. Before use, a stock solution (5 mg $mL^{-1}$ in $H_2O$) was prepared.

*BBR purification and quantification*

Purification of the crude *Rhizoma coptidis* extract was performed by semipreparative HPLC. Firstly, the extract was fractionated using a gradient of MeCN (15–65%) for 30 min and all the fractions were collected. After identifying the fractions that showed POP inhibitory activity ($t_R = 18$ min), these were freeze-dried and purified by semipreparative HPLC using an isocratic gradient of MeCN (28%) during 30 min. The active fractions with a purity of more than 99% were selected which corresponded to a $t_R$ of 12 min. Analytical data for BBR are as follows: $^1$H NMR ([D$_6$]DMSO, 400.125 MHz): $\delta$ 2.48 (t, 2H, $J = 6.2$ Hz), 4.05 (s, 3H), 4.08 (s, 3H), 4.92 (t, 2H, $J = 6.2$ Hz), 6.157 (s, 2H), 7.07 (s, 1H), 7.78 (s, 1H), 7.99 (d, 2H, $J = 8.8$ Hz), 8.12 (d, 1H, $J = 9.0$ Hz), 8.93 (s, 1H), 9.88 (s, 1H); $^{13}$C NMR ([D$_6$]DMSO 100.611 MHz): $\delta$ 27.0, 55.9, 57.7, 62.6, 102.8, 106.1, 109.1, 120.9, 121.1, 122.1, 124.2, 127.4, 131.4, 133.6, 138.2, 144.3, 146.1, 148.4, 150.5, 151.1.

Quantification of BBR in the plant extract was done by RP-HPLC $C_{18}$ using a calibration curve. Therefore, different solutions of BBR were prepared (1–100 μM). These solutions were analyzed using a gradient of MeCN (15–65%) in $H_2O$ during 15 min at 230 nm (BBR $t_R = 8.7$).

*Expression and purification of POP*

POP was obtained by expression in *E. coli* and affinity purification using a His tail fusion as reported previously.[26]

*POP activity assays*

*Experiments using ZGP-AMC*

POP activity was determined following the method described by Toide et al.[13] The reactions were performed in 96 well microtiter plates, which allowed the simultaneous monitoring of multiple reactions. For each reaction, activity buffer (131 μL, 100 mM of Na/K phosphate buffer, pH 8.0) was preincubated for 15 min at 37 °C with POP (7 nM) and with the corresponding BBR solution (3 μl). A BBR stock solution was prepared in DMSO (100 mM), dilutions were prepared from the stock solution with DMSO. A control with the same DMSO concentration was performed. After preincubation, ZGP-AMC (10 μl, 3 mM in 40% of 1,4-dioxane) was added and the reaction was incubated for 1 h at 37 °C. The reaction was stopped with sodium acetate (150 μl, 1 M, pH 4) and the formation of AMC was measured fluorometrically. The excitation and emission wavelengths were 360/40 and 485/20 nm, respectively. The $IC_{50}$ value was defined as the concentration of inhibitor required to inhibit 50% of POP activity.

*Experiments using $^{19}$F substrate, ZGPF-4-CF$_3$*

A previously reported $^{19}$F NMR POP inhibition assay was used.[26] Briefly, the assay is based on the use of the fluorine-labeled POP substrate, Z-Gly-Pro-Phe-4($CF_3$)-$NH_2$ (ZGPF-4-$CF_3$), which allows the monitoring of POP inhibitory activity by means of $^{19}$F NMR spectroscopy and circumvents the false positive or false negative problems associated with commonly used fluorimetric and colorimetric assays. The final concentration of TCMs in the assay buffer was 100 μg mL$^{-1}$ (14 μl of a TCM stock at 5 mg mL$^{-1}$ was added to the reaction mixture).

*Trypsin inhibition assay*

Trypsin inhibitory activity assay was performed in 96 well microtiter plates. Bovine trypsin (10 μl of a 10 ng/μl stock) was preincubated for 15 min at 37 °C with the corresponding BBR solution (3 μl) in activity buffer (125 μl, 20 mM of Tris-HCl pH 8.0). A BBR stock solution was prepared in DMSO (100 mM), dilutions were prepared from the stock with DMSO and added to the final concentrations of 400 μM, 300 μM, 200 μM, and 100 μM. After preincubation, Bz-Arg-AMC-HCl (10 μl, 3 mM in 40% of 1,4-dioxane) was added and the reaction was incubated for 1 h at 37 °C. The reaction was stopped with sodium acetate (150 μl, 1 M, pH 4) and the formation of AMC was measured fluorometrically.

Abbreviations

SZ: schizophrenia; BD: bipolar affective disorder; POP: prolyl oligopeptidase; TCM: traditional Chinese medicine; BBR: berberine; CNS: Central Nervous System; BBB: Blood-Brain Barrier; Z: benzyloxycarbonyl; AMC: 7-amino-4-methylcoumarin; TFA: trifluoroacetic acid; HPLC: high performance liquid chromatography; MeCN: acetonitrile; DMSO: dimethylsulfoxide; BAPNA: Nα-Benzoyl-DL-arginine-p-nitroaniline

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**Keywords:** berberine · fluorine NMR spectroscopy · prolyl oligopeptidase · schizophrenia · traditional Chinese medicine

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