Received: 24 April 2022 | Revised: 16 March 2023 | Accepted: 21 March 2023

DOI: 10.1002/mrc.5348

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LETTER - SPECTRAL ASSIGNMENT

A new amide from the fruiting bodies of *Tricholoma bakamatsutake*

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Funding information
Yunnan Key Project of Science and Technology, Grant/Award Number: 202202AE090001; Postdoctoral Directional Training Foundation of Yunnan Province, Grant/Award Number: E23174K2; Postdoctoral Research Funding Projects of Yunnan Province, Grant/Award Number: E2313442

Abstract

A new amide tricholomine C was isolated from the dried fruiting bodies of *Tricholoma bakamatsutake*. Its structure was identified by a combination of nuclear magnetic resonance spectroscopic analysis and electronic circular dichroism (ECD) calculations. The ethyl alcohol crude extract and tricholomines A–C from T. bakamatsutake were evaluated for neuroprotective activities. Of these substances, the crude extract showed weak neurite outgrowth-promoting activity in rat pheochromocytoma (PC12) cells, as well as weak inhibitory activities against acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE).

INTRODUCTION

Tricholoma bakamatsutake is a wild edible mushroom that belongs to the order Basidiomycota, class Agaricales, and family Tricolomataceae. "Bakamatsutake" originated in the Japanese dialect, translated into English as "foolish pine-mushroom."[1–4] It is mainly distributed in New Guinea,[1] Japan,[2] Korea,[3] and China's Jilin, Sichuan, Yunnan province.[4] So far, the research on *T. bakamatsutake* has mainly focused on taxonomy and phylogeny.[5–14] To date, More than 100 different secondary metabolites have been isolated from the fruiting bodies of *Tricholoma* species, they include pulvinic acid derivatives, ergostane steroids, phenols, indoles, and most triterpenoids.[4]

Studies on the chemical composition and bioactive compounds of *T. bakamatsutake* are very limited.[15,16] In our previous chemical analysis, we isolated two new amides tricholomines A and B[17] from fruitbodies of

T. bakamatsutake. In this paper, we report on the isolation and structural elucidation of a new amide, tricholomine C.

RESULTS AND DISCUSSION

Tricholomine C was obtained as a brown solid and its molecular formula was shown to be $C_{15}H_{23}NO_4$, with five pairs of hydrogen deficiencies, by high-resolution electrospray ionization mass spectrometry (HR-ESI-MS) at *m/z* 304.1519 $[M + Na]^+$ (calcd for $C_{15}H_{23}NO_4Na$, 304.1519) and its ^{13}C nuclear magnetic resonance (NMR) data (Table 1). The infrared (IR) spectrum indicated the presence of hydroxy (3443 cm^{-1}) and carbonyl (1,650 cm^{-1}) groups. The ^1H NMR data for **1** revealed the presence of a hydroxy group [δ_H 4.49 (1H, d, $J = 10.8$ Hz)], a methoxy group [δ_H 3.29 (3H, s)], and two methyl groups [δ_H 1.97 (3H, s) and 1.42 (1H, d, $J = 7.0$ Hz)]. The ^{13}C NMR data identified 15 carbon signals, which showed the presence of two carbonyl groups (δ_C 202.7 and 174.8), two sp^2 quaternary carbons (δ_C 159.6 and 133.4), three sp^3 methines (δ_C 90.4, 72.9 and 52.4), five sp^3 methylenes (δ_C 38.3, 33.5, 29.3, 24.6 and 21.7), and three methyls (δ_C 53.9, 21.5 and 14.4).

TABLE 1 ^1H (500 MHz) and ^{13}C (125 MHz) nuclear magnetic resonance data of compound **1** in $CDCl_3$ (δ in ppm, J in Hz).

No.	δ_C	δ_H
1	14.4	1.42 (d, 7.0)
2	52.4	4.18 (dq, 8.7, 7.0)
3	72.9	4.59 (dd, 10.8, 8.7)
4	133.4	
5	159.6	
6	33.5	2.30 (m) 2.34 (dd, 9.1, 3.8)
7	21.7	1.88 (m) 1.91 (dd, 3.8, 2.3)
8	38.3	2.32 (m) 2.52 (ddd, 16.6, 6.9, 5.3)
9	202.7	
10	21.5	1.97 (s)
2′	174.8	
3′	29.3	2.19 (ddd, 16.9, 9.8, 3.2) 2.41 (m)
4′	24.6	1.90 (m) 2.03 (m)
5′	90.4	4.75 (dd, 6.1, 1.4)
6′	53.9	3.29 (s)
3-OH		4.49 (d, 10.8)

According to the correlations in the COSY spectrum (Figure 1), three fragments, C-1-C-2-C-3, C-6-C-7-C-8, and C-3′-C-4′-C-5′ were confirmed. Based on heteronuclear multiple bond coherence (HMBC) correlations (Figure 1) from H-1 to C-3, H-2 to C-4, C-2′, and C-5′; H-3 to C-1, C-5, and C-9; H-6 to C-4, C-8, and C-10; H-7 to C-5 and C-9; H-8 to C-4 and C-6; and H-10 to C-4 and C-6, a 3-methylcyclohex-2-enone was deduced, with one double bond of $\Delta^{4(5)}$, one carbonyl group of C-9, and one methyl group at C-5. According to its molecular formula and HMBC correlations from H-3′ to C-5′; H-4′ to C-2′; H-5′ to C-2′, C-3′, and C-6′; and H-6′ to C-5′, a methoxy group at C-5′, a 5′-methoxy butyrolactam consisting N-C-2′-C-3′-C-4′-C-5′ were elucidated. One methyl group at C-2, a hydroxy group at C-3 were comfirmed. Thus, the rather constitution of compound **1** was deduced as shown in Figure 1.

The stereostructure of **1** was established as 2*R*,3*R*,5′*S*, according to the VCD calculations analysis (Supporting Information). Meanwhile, the calculated ECD spectrum of 2*R*,3*R*,5′*S* matched the experimental ECD spectrum of Compound **1** by comparison of the experimental and calculated ECD spectra of **1** (Figure 2) and named tricholomine C as (*S*)-1-((1*R*,2*R*)-1-hydroxy-1-(2-methyl-6-oxocyclohex-1-en-1-yl)propan-2-yl)-5-methoxypyrrolidin-2-one.

The ethyl alcohol crude extract and tricholomines A–C were evaluated for neuroprotective activities. As shown in Tables 2 and 3, the crude extract (20 μg/mL) showed weak neurite outgrowth-promoting activity in rat pheochromocytoma (PC12) cells, as well as weak inhibitory activities against acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE), with rates from 9.58% to 33.36%. Tricholomines A–C were inactive.

EXPERIMENTAL

General experimental procedures

Optical rotations were recorded using a JASCO P-1020 polarimeter (JASCO Corp., Tokyo, Japan). UV spectra were taken on a Shimadzu UV-2401 PC spectrophotometer (Shimadzu, Kyoto, Japan). ECD spectra were recorded on a Chirascan CD spectrometer (Applied Photophysics Ltd., Leatherhead, UK). IR spectra were measured on a Bruker Tensor 27 FTIR Spectrometer (Bruker Corp., Ettlingen, Germany) with KBr disks. The ^1H and ^{13}C NMR spectra were obtained on Bruker DRX-500 spectrometers (Bruker Bio-Spin GmbH, Rheinstetten, Germany) with TMS as an internal standard. ESI-MS and

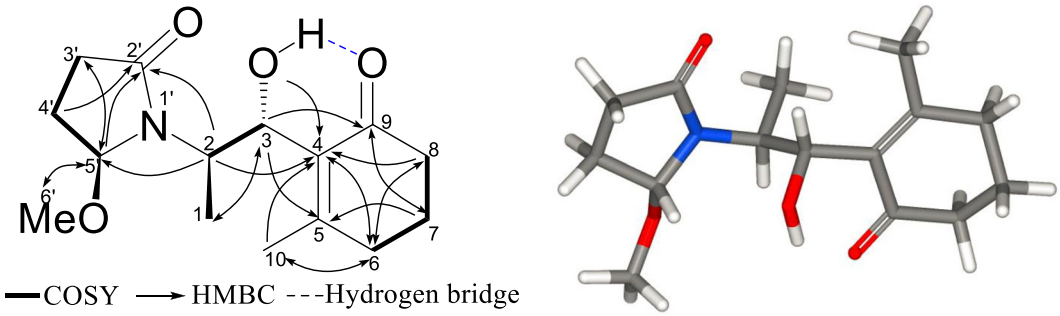

FIGURE 1 The stereostructure and key 2D nuclear magnetic resonance correlations of compound **1**. COSY, correlation spectroscopy; HMBC, heteronuclear multiple bond coherence.

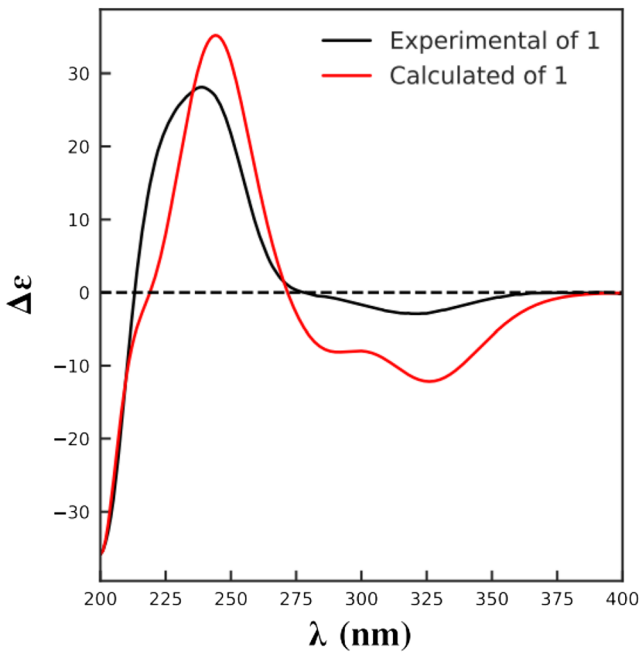

FIGURE 2 Comparison of the experimental and calculated electronic circular dichroism spectra of **1**.

TABLE 2 The effect of the crude extract and compounds from *Tricholoma bakamatsutake* on neurite outgrowth-promoting activity in PC12 cells.

	Concentration	72 h Promoting activity (%)
Tricholomine A	10 μM	5.09
Tricholomine B	10 μM	5.54
Tricholomine C	10 μM	3.83
Crude extract	20 μg/mL	9.59
Positive NGF	50 ng/mL	18.83
Negative NGF	5 ng/mL	4.32
Blank	—	None

Abbreviation: NGF, nerve growth factor.

HR-ESI-MS were carried out on an API QSTAR Pulsar 1 mass spectrometer (Applied Biosystems/MDS Sciex, Foster City, CA, USA). Silica gel G (80–100 and 300–400 mesh, Qingdao Meigao Chemical Co., Ltd., Qingdao, People's Republic of China), C_{18} silica gel (40–75 μm, Fuji Silysia Chemical Ltd., Aichi, Japan), and Sephadex LH-20 (GE Healthcare BioSciences AB, Uppsala, Sweden) were used for column chromatography. TLC zones were visualized under UV light at 254 nm and by dipping into 5% H_2SO_4 in alcohol followed by heating.

NMR spectroscopic methods

All NMR experiments (^{1}H-NMR, ^{13}C-NMR, DEPT135, ^{1}H-^{1}H COSY, heteronuclear single quantum coherence [HSQC], HMBC, and rotating frame overhauser effect spectroscopy [ROESY]) were recorded on a Bruker Ultrashield TM Plus 500 MHz spectrometer equipped with 5 mm probes. Compound **1** was dissolved in 0.5 mL $CDCl_3$ as the solvent, which contained 0.03% TMS as an internal standard. The ^{1}H- and ^{13}C-NMR spectra (at 500 and 125 MHz, respectively) were measured at a temperature of 296.1 K. Chemical shifts (δ) were given in parts per million, and coupling constants (J) were reported in Hertz. Data processing was carried out with TOPSPIN 3.2 version (Bruker, Germany). The pulse conditions were as follows: for the ^{1}H-NMR spectra, 90 (10.80 μs) flip angle at 0 dB, acquisition time (AQ) = 3.276 s, relaxation delay (RD) = 1.0 s, spectral width (SW) = 10,000 Hz, time domain data points (TD) = 65,536, and digital resolution = 0.153 Hz per point; for the ^{13}C-NMR spectra, 90 (15.07 μs) flip angle at 0 dB, AQ = 1.101 s, RD = 4.5 s, SW = 29761.9 Hz, TD = 65,536, and digital resolution = 0.454 Hz per point; and for the DEPT 135 spectra, 90 (15.07 μs) flip angle at 0 dB, AQ = 1.10 s, RD = 4.5 s, SW = 29761.9 Hz, TD = 65,536, and digital resolution = 0.454 Hz per point. The ^{1}H-^{1}H COSY

TABLE 3 The inhibitory effect of the crude extract and compounds from *Tricholoma bakamatsutake* on acetylcholinesterase and butyrylcholinesterase.

	Concentration	AChE inhibition (%)	BuChE inhibition (%)
Tricholomine A	50 μM	3.85 ± 2.26	5.00 ± 0.80
Tricholomine B	50 μM	0.22 ± 1.33	3.56 ± 2.21
Tricholomine C	50 μM	15.84 ± 0.62	9.55 ± 2.02
Crude extract	20 μg/ml	33.36 ± 2.37	24.09 ± 0.54
Tacrine	0.333 μM	64.88 ± 0.96	92.26 ± 0.25

Note: Tacrine was a positive control.

spectra were obtained using the following conditions: AQ = 0.102 s, RD = 1.0 s, SW = 5000 (F1) and 5000 Hz (F2), SI = 1024 (F1), and 1024 (F2). The HSQC spectra were obtained using the following conditions: AQ = 0.1024 s, RD = 1.2 s, SW = 5000 (F1) and 5000 Hz (F2), and SI = 1024 (F1) and 1024 (F2). The HMBC spectra were obtained using the following conditions: AQ = 0.1204 s, RD = 1.8 s, SW = 20,406 (F1) and 5000 Hz (F2), SI = 1024 (F1) and 2048 (F2), and *J* (XH) long range = 8.0. The ROESY spectra were obtained using the following conditions: AQ = 0.1024 s, RD = 1.2 s, SW = 4998.5 (F1) and 5000 Hz (F2), and SI = 1024 (F1) and 1024 (F2).

Fungal material

The dried fruiting bodies of *T. bakamatsutake* were bought from Ningqiang County, Shanxi Province, China, in October 2015. A voucher specimen (no. FM0881) was identified as *T. bakamatsutake* by Dr. Fu-Qiang Yu and has been deposited at the Kunming Institute of Botany, Chinese Academy of Sciences.

Extraction and isolation

The dried fruiting bodies of *T. bakamatsutake* (2.5 kg) were pulverized and macerated five times with 90% EtOH (total 10 L) at room temperature. Evaporation of the solvent provided an EtOH extract (300.9 g; 12.0% yield). The EtOH extract was dissolved in water and partitioned into petroleum ether, ethyl acetate, and *n*-butanol in turn. The EtOAc-soluble fraction was subjected to normal-phase silica gel column chromatography with a gradient of petroleum ether/acetone (20:1 to 1:1, v/v)], to obtain five fractions (Fr. A–E). Fr. C (1.8 g) was fractionated by RP C_{18} CC ($MeOH/H_2O$, 30:70 to 100:0) to obtain five subfractions (Fr. C1 − C5): Fr. C2 (290.0 mg) was purified by Sephadex LH-20 (MeOH) and then subjected to normal silica gel CC (300–400 mesh; petroleum ether/acetone, 10:1, v/v) to yield tricholomine C (1.2 mg).

Tricholomine C

Brown solid, $[\alpha]^{26.2}_{D}$ + 9.1 (*c* 0.34, MeOH); UV (MeOH) λ_{max} (log ε) 242 (3.50), 202 (3.31) nm; ECD (*c* 0.51, MeOH) λ_{max} ($\Delta\varepsilon$) 399 (−0.06), 318 (−0.86), 239 (+8.52), 199 (−10.89) nm; ^1H and ^{13}C NMR data, see Table 1; ESI-MS *m/z* 304 $[M + Na]^+$, HR-ESI-MS *m/z* 304.1519 $[M + Na]^+$ (calcd for $C_{15}H_{23}NO_4$, 304.1519).

Neurite outgrowth-promoting activity assay

The neurotrophic activities of the test compounds were examined according to an assay using PC12 cells as reported.[18] Briefly, PC12 cells were maintained in 1640 medium supplemented with 10% horse serum (HS) and 5% fetal bovine serum (FBS) and incubated at 5% CO_2 and 37°C. Test compounds were dissolved in dimethyl sulfoxide (DMSO). For the neurite outgrowth-promoting activity bioassay, PC12 cells were seeded at a density of 5×10^4 cells/mL in a 48-well plate coated with poly-L-lysine. After 24 h, the medium was changed to that containing 10 μM of each test compound or 20 μg/mL of crude extract plus 5 ng/mL nerve growth factor (NGF) or various concentrations of NGF (50 ng/mL for the positive control, 5 ng/mL for the negative control). The final concentration of DMSO was 0.05%, and the same concentration of DMSO was added to the negative control. After 72 h incubation, the neurite outgrowth was assessed under a phase contrast microscope. Neurite processes with a length equal to or greater than the diameter of the neuron cell body were scored as neurite-bearing cells. The ratio of the neurite-bearing cells to total cells (with at least 100 cells examined/view area; five viewing area/well) was determined and expressed as a percentage.

Acetylcholinesterase/butyrylcholinesterase inhibitory activity assay

Acetylcholinesterase/butyrylcholinesterase (AChE/BuChE) inhibitory activity of the compounds isolated was assayed by the spectrophotometric method developed by Ellman et al[19] with slight modification. *S*-Acetylthiocholine iodide, *S*-butyrylthiocholineiodide, 5,5′-dithio-bis-(2-nitrobenzoic) acid (DTNB, Ellman's reagent), AChE and BuChE derived from human erythrocytes were purchased from Sigma-Aldrich Company. Compounds were dissolved in DMSO. The reaction mixture (total 200 μL) containing phosphate buffer (pH 8.0), test compound (20 μg/mL) and crude extract (50 μM), and AChE (0.02 U/mL) or BuChE (0.016 U/mL) was incubated for 20 min (37°C). Then, the reaction was initiated by the addition of 40 μL of a solution containing DTNB (0.625 mM) and acetylthiocholine iodide (0.625 mM) or butyrylthiocholine iodide (0.625 mM) for AChE or BuChE inhibitory activity assay, respectively. The hydrolysis of acetylthiocholine or butyrylthiocholine was monitored at 405 nm every 30 s for 1 h. Tacrine was used as a positive control with a final concentration of 0.333 μM. All these actions were performed in triplicate. The percentage inhibition was calculated as follows: % inhibition = (E − S)/E × 100 (E is the activity of the enzyme without test compound, and S is the activity of the enzyme with test compound).

ACKNOWLEDGMENTS

This work was supported by Yunnan Key Project of Science and Technology (202202AE090001), Postdoctoral Directional Training Foundation of Yunnan Province (E23174K2), and Postdoctoral Research Funding Projects of Yunnan Province (E2313442). We thank Guangzhou Yinfo Information Technology Co., Ltd., for providing a friendly and versatile web server (https://cloud.yinfotek.com) to aid the docking studies.

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PEER REVIEW

The peer review history for this article is available at https://www.webofscience.com/api/gateway/wos/peer-review/10.1002/mrc.5348.

REFERENCES

[1] Y. Otani, *Trans. Mycol. Soc. Japan* **1976**, *17*(3–4), 363.
[2] H. Shimazono, Y. Ogata, T. Yasunaga, H. Kawaguchi, *Trans. Mycol. Soc. Japan* **1979**, *20*(2), 185.
[3] K. H. Ka, S. M. Jeon, R. Ryoo, J. A. Kang, K. S. Hong, *Korean J. Med. Mycol.* **2015**, *43*(2), 125.
[4] W. Yun, I. R. Hall, L. A. Evans, *Econ. Botany* **1997**, *51*(3), 311.
[5] M. Gong, J. Cao, L. Su, F. Wang, Y. Chen, Y. Chen, *Forest Res.* **1999**, *12*, 31.
[6] X. L. Quan, T. Y. Shi, M. S. Zhang, *Edible Fungi* **2009**, *31*(6), 14+21.
[7] X. L. Quan, W. J. Fu, J. R. Wu, *Edible Fungi* **2006**, *(2)*, 21.
[8] F. Q. Yu, PhD thesis, Kunming Institute of Botany, Chinese Academy of Sciences, China **2007**.
[9] M. Hitoshi, O. Yuko, Y. Muneyoshi, Y. Akiyoshi, S. Katahata, Y. Otsuka, K. Babasaki, H. Neda, *Mycorrhiza* **2013**, *23*(6), 447.
[10] X. Lan, S. Q. Wu, M. Y. Jin, X. L. Quan, *Jiangsu J. Agri. Sci.* **2015**, *31*(4), 906.
[11] X. Lan, M. L. Quan, M. Y. Jin, S. Wu, *Acta Edulis Fungi* **2015**, *22*(1), 21.
[12] J. W. Hou, Master thesis, Shanxi University, China **2020**.
[13] H. Deng, Z.M. Cao, Y.J. Yao. Review of research work on *Tricholoma bakamatsutake* in China. Proceedings of the Sixth National Symposium on Mycology. **2003**: 132–136.
[14] Z. Z. Deng, *Agric. Sci. Technol. Equip.* **2019**, 290(2), 19.
[15] X. L. Quan, H. J. Wang, T. Y. Shi, M. S. Zhang, *Edible Fungi* **2007**, *2*, 54.
[16] S. Q. She, G. Liu, X. Y. Du, W. S. Gao, X. H. Quan, D. Z. Kang, *J. Med. Sci. Yanbian Univ.* **2014**, *37*(2), 117.
[17] F. M. Zhang, Y. H. Wang, F. Q. Yu, Y. Hua, *Magn. Reson. Chem.* **2021**, *59*(5), 587.
[18] L. A. Greene, A. S. Tischler, *Proc. Natl. Acad. Sci. U. S. A.* **1976**, *73*, 2424.
[19] G. L. Ellman, K. D. Courtney, V. J. Andres, R. M. Featherstone, *Biochem. Pharmacol.* **1961**, *7*, 88.

SUPPORTING INFORMATION

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How to cite this article: F.-M. Zhang, B. Lu, Y.-H. Wang, F.-Q. Yu, *Magn Reson Chem* **2023**, *61*(7), 443. https://doi.org/10.1002/mrc.5348