

Calcium Sensitizers Isolated from the Edible Pine Mushroom, *Tricholoma matsutake* (S. Ito & Imai) Sing.

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Three lactam compounds were isolated from the fruiting body of *Tricholoma matsutake* (S. Ito & Imai) Sing., an edible mushroom, and their structures were identified as *cyclo-S-proline-R-leucine* (**1**), hexahydro-2*H*-azepin-2-one (**2**), and butyl 5-oxo-2-pyrrolidine carboxylate (**3**) by chemical, physicochemical, and spectral evidence. In *in vitro* screening tests, compounds **1** and **2** acted as calcium sensitizers in ventricular cells from rat. Further studies on compounds **1** and **2** in *ex vivo* isolated right atria showed positive inotropic effects without disturbing the spontaneous beating rate. The inotropic effect of compounds **1** and **2** could be greatly abolished by pretreating the myocardium in Ca²⁺-free solution. These findings indicate that compounds **1** and **2** can significantly increase the calcium ion concentration ([Ca²⁺]_i) in myocytes, which is greatly dependent on the influx of extracellular Ca²⁺.

Key words: *Tricholoma matsutake* Sing., Lactam Compounds, Positive Inotropic Effect

Introduction

Classic inotropic agents induce intracellular calcium flux in myocardial cells and thereby contractility. Thus, application of these drugs is theoretically appealing as a treatment to increase the cardiac contractility and improve the cardiac output in heart failure patients. Although patients benefit from the improvement of the symptoms of heart failure, the administration of inotropic agents remains controversial to date. Some agents have been associated with critical negative side effects including increased myocardial oxygen consumption, cardiac arrhythmias, and mortality in a variety of clinical settings. On the other hand, there is evidence suggesting that selected patients may benefit from properly chosen inotropic agents. For example, the short-term use of milrinone not only enhances the cardiac ejection but also improves inflammatory and apoptotic biomarkers (Lanfear *et al.*, 2009). Levosimendan, a new calcium sensitizer and K_{ATP} channel opener, which increases the cardiac contractility without changing the intracellular calcium availability, has altered the perception of the use of inotropes in acute heart failure, and has demonstrated more favourable potential therapeutic effects compared with classic inotropic agents such as car-

diac glycosides or beta-agonists (Fotbolcu and Duman, 2010). In this case, a calcium sensitizer can increase the intracellular calcium load but not increase the oxygen consumption. Hence, in our opinion, the use of positive inotropic agents is not detrimental *per se*. As long as the development of suitable inotropic agents and their proper administration can be achieved, an inotropic therapy will be valuable and significant in heart failure.

The pine mushroom, *Tricholoma matsutake* (S. Ito & Imai) Sing., is an edible fungus, which is mainly distributed in China and is a highly valued edible mushroom all over the world. *T. matsutake* has been reported to have excellent biological activities, such as enhancing health, alleviating pain, and dissipating phlegm (Guerin-Laguette *et al.*, 2003). *T. matsutake* contains a variety of constituents, such as polysaccharides, proteins, fat, vitamins, and a multitude of amino acids, in particular eight essential ones. Polysaccharides are the major bioactive compounds in *T. matsutake*, like in other mushrooms, displaying antidiabetic, anticancer, and antihyperlipidemic activities, among others. Potential immunostimulatory effects have been demonstrated in several studies (Kim *et al.*, 2008; Byeon *et al.*, 2009), but little studies have been carried out on the chemical constituents of *T. matsutake*.

In this paper, we report three lactam compounds obtained from *T. matsutake* which were identified as *cyclo-S*-proline-*R*-leucine (**1**), hexahydro-2*H*-azepin-2-one (**2**), and butyl 5-oxo-2-pyrrolidine carboxylate (**3**) on the basis of spectral analyses, including MS, ¹H NMR, ¹³C NMR, DEPT, and HMBC. Compounds **1–3** have been isolated before from plants (Marchal, 1982; Tang *et al.*, 2001; Chu *et al.*, 2006). Further studies on the actions of compounds **1** and **2** in *ex vivo* right atria revealed positive inotropic effects without disturbance of the spontaneous beating rate. The inotropic effect of compounds **1** and **2** could be largely reduced by pretreating the myocardia in Ca²⁺-free solution.

Experimental

Fungal material and chemicals

Tricholoma matsutake (S. Ito & Imai) Sing. was collected in Kun Ming, Yunnan Province, P. R. China, and identified by Dr. Jincai Lu, Shenyang Pharmaceutical University, Shenyang, P. R. China. A voucher specimen was deposited at the School of Pharmacy, Harbin Medical University, Harbin, P. R. China. All chemicals used were of analytical grade and were purchased from Shandong Yuyang Industry Co., Ltd., Shandong, P. R. China.

Analytical procedures

¹H and ¹³C NMR spectra were recorded with a JNM-LA500 spectrometer (JEOL, Tokyo, Japan) at 500 and 125 MHz, respectively. HR-ESI- and EI-mass spectra were obtained on Bruker (Billerica, MA, USA) APEX microTOF-Q and JEOL GC mass spectrometers, respectively. Optical rotation was measured with a Perkin-Elmer (Fremont, CA, USA) 241 polarimeter. HPLC was performed with a Shimadzu (Tokyo, Japan) RID-6A refractive index detector, the laser scanning confocal microscope Fluoview-FV300 was from Olympus (Tokyo, Japan).

Extraction and isolation

The air-dried fruiting bodies (5 kg) of *T. matsutake* were extracted with 70% EtOH (50 L) at room temperature for 8 weeks without stirring. The ethanolic extract was partitioned between EtOAc and H₂O. The EtOAc-soluble portion (19.1 g) was repeatedly subjected to chromatography on a silica gel column (30 g) which was

eluted with a CHCl₃/MeOH gradient from 80:1 (v/v) to 5:1 to afford seven fractions: fraction 1 (0.69 g), fraction 2 (1.43 g), fraction 3 (0.26 g), fraction 4 (1.42 g), fraction 5 (2.40 g), fraction 6 (4.7 g), and fraction 7 (8.7 g). Fraction 5 was further subjected to a reversed phase chromatography (ODS) column (300 g) eluted with a MeOH/H₂O gradient (35%, 45%, 55%, v/v) to afford three fractions: fraction 5-1 (0.1 g), fraction 5-2 (0.06 g), and fraction 5-3 (1.4 g). Fraction 5-1 was further purified by preparative HPLC [column, 5C18-MS-II, 4.6 x 250 mm; mobile phase, MeOH/H₂O (15:85); flow rate, 8 mL/min] to give compound **3** (37 mg). Fraction 5-2 was further purified by preparative HPLC [mobile phase, MeOH/H₂O (25:75); flow rate, 8 mL/min] to give compound **1** (31 mg). Fraction 5-3 was further purified by preparative HPLC [mobile phase, MeOH/H₂O (30:70); flow rate: 8 mL/min] to give compound **2** (31 mg).

The structures of compounds **1–3** were elucidated by comparing their ¹H, ¹³C NMR, and mass spectroscopic data with those reported in the literature.

Calcium ion concentration ([Ca²⁺]_i) measurement

Male Wistar rats (weighting 250 ~ 300 g) were provided by the animal center of the Second Affiliated Hospital of Harbin Medical University, Harbin, P. R. China. [Ca²⁺]_i fluorescence measurements in cardiomyocytes followed the procedure of Rothstein *et al.* (2005). The effects of compounds **1** and **2** on the level of [Ca²⁺]_i in single myocytes were measured. KCl and Tyrode's solution, containing less than 0.3% dimethyl sulfoxide (DMSO), were used as positive and negative controls, respectively. Briefly, isolated ventricular myocytes were adhered to the cover slips of the chamber and incubated with a solution containing Fluo-3/AM (20 mM) and Pluronic F-127 (0.03%) at 37 °C for 45 min. After loading, extracellular Fluo-3/AM was removed by washing the myocytes once with Tyrode's solution. Fluorescence changes of the Fluo-3/AM-loaded cells were detected by a laser scanning confocal microscope (excitation at 488 nm from an Ar ion laser; emission at 530 nm). **1** and **2** (5.64 and 10.36 mM, respectively) were added between the 3rd and 4th image. The fluorescence intensities before (*F*₀) and after (*F*_{max}) drug administration were recorded and the images were stored in disks. The change of [Ca²⁺]_i

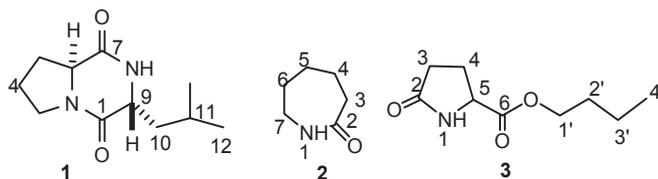


Fig. 1. The chemical structures of *cyclo-S-proline-R-leucine* (**1**), *hexahydro-2H-azepin-2-one* (**2**), and *butyl 5-oxo-2-pyrrolidine carboxylate* (**3**) isolated from *Tricholoma matsutake* (S. Ito & Imai) Sing.

was represented by the ratio of fluorescence intensity of Fluo-3/AM over base (F_{\max}/F_0).

Measurement of the spontaneous beating rate and contractile force of the isolated heart muscle

The Wistar rat was sacrificed by a blow on the head, and the heart was rapidly removed (Sun *et al.*, 2006). The right atrium was quickly mounted vertically in a 5-mL organ bath filled with a physiological solution, gased with 95% O₂/5% CO₂, containing NaCl (118 mM), KCl (4.7 mM), MgSO₄ (1.1 mM), KH₂PO₄ (1.2 mM), CaCl₂ (1.5 mM), NaHCO₃ (25 mM), and glucose (10 mM), pH 7.35–7.45. One end of the tissue was secured to the tissue holder, and the other end was connected to a force transducer by a silk thread. The right atrium beat spontaneously in this condition. The resting tension of the tissue was set at 0.2 g, the tissue was equilibrated for 30 min, and the solution in the organ bath was changed every 20 min. **1** (1.69, 3.38, 6.76, 13.52, 27.04 mM, content of DMSO less than 0.3‰) and **2** (3.11, 6.22, 12.44, 24.88, 49.76 mM, content of DMSO less than 0.3‰) were cumulatively added into the organ bath with 3-min intervals, while physiological solution containing the same concentration of DMSO was added in the same way to the control groups. Contractile force and spontaneous beating rate of the right atrium were recorded by a force transducer (MedLab BL-420E+ recording system; TaiMeng, Chengdu, China).

Results and Discussion

Effects of **1** and **2** on $[Ca^{2+}]_i$ of ventricular myocytes

For many decades, lactams have been studied and developed as antibiotics. As studies went on, the pharmacological actions of lactams were found to be manifold, such as neuroprotection, inhibitory effect on platelet activation, and modula-

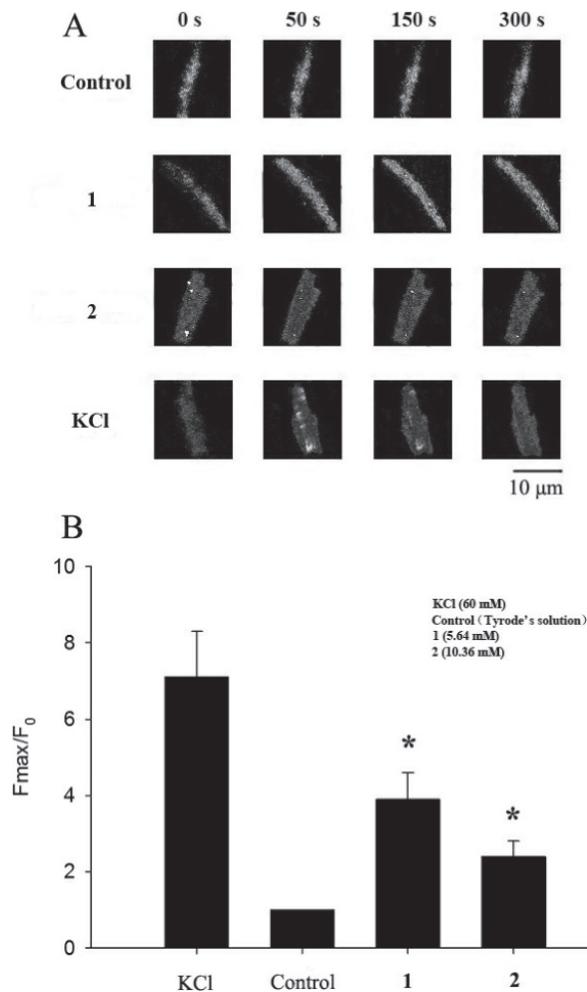


Fig. 2. Effects of **1** and **2** on $[Ca^{2+}]_i$ in Wistar rat ventricle myocytes observed under a laser scanning confocal microscope. (A) The images of single cardiomyocytes were scanned from 0 s to 300 s. KCl was used as positive control. (B) The change of $[Ca^{2+}]_i$ is represented as the ratio of the fluorescence intensity of Fluo-3/AM (F_{\max} and F_0 mean the fluorescence intensities of peak and resting state, respectively). Data are expressed as mean \pm SD, $n = 4$; * $p < 0.05$ versus control group.

tion of sodium channel inactivation (Rothstein *et al.*, 2005; Pavanetto *et al.*, 2007; Jones *et al.*, 2009).

In the present study, tentative confocal microscopic tests were conducted to observe the modulation of $[Ca^{2+}]_i$ by compounds **1** and **2** (Fig. 1) in ventricle myocytes, and the intriguing result was that the two compounds were able to induce an elevation of the intracellular calcium content. Fig. 2A shows the images of $[Ca^{2+}]_i$ in differently treated single myocytes by laser scanning confocal microscopy at 0, 50, 150, and 300 s. Fig. 2B presents the relative fluorescence intensity (F_{max}/F_0) in the myocytes. Compared with the control group, both **1** and **2** provoked an increase in $[Ca^{2+}]_i$ in ventricle

myocytes, however, with different potency. At the same concentration of the compounds, the level of F_{max}/F_0 was enhanced twofold by **1** in comparison with **2**. The mechanism of the increase of $[Ca^{2+}]_i$ induced by **1** and **2** is not clear, and the different potencies of the two compounds may reflect differences in the receptor affinities.

Effects of 1 and 2 on the contractile force and spontaneous beating rate of the isolated heart muscle

The elevation of $[Ca^{2+}]_i$ by **1** and **2** in isolated ventricle myocytes might be due to a positive

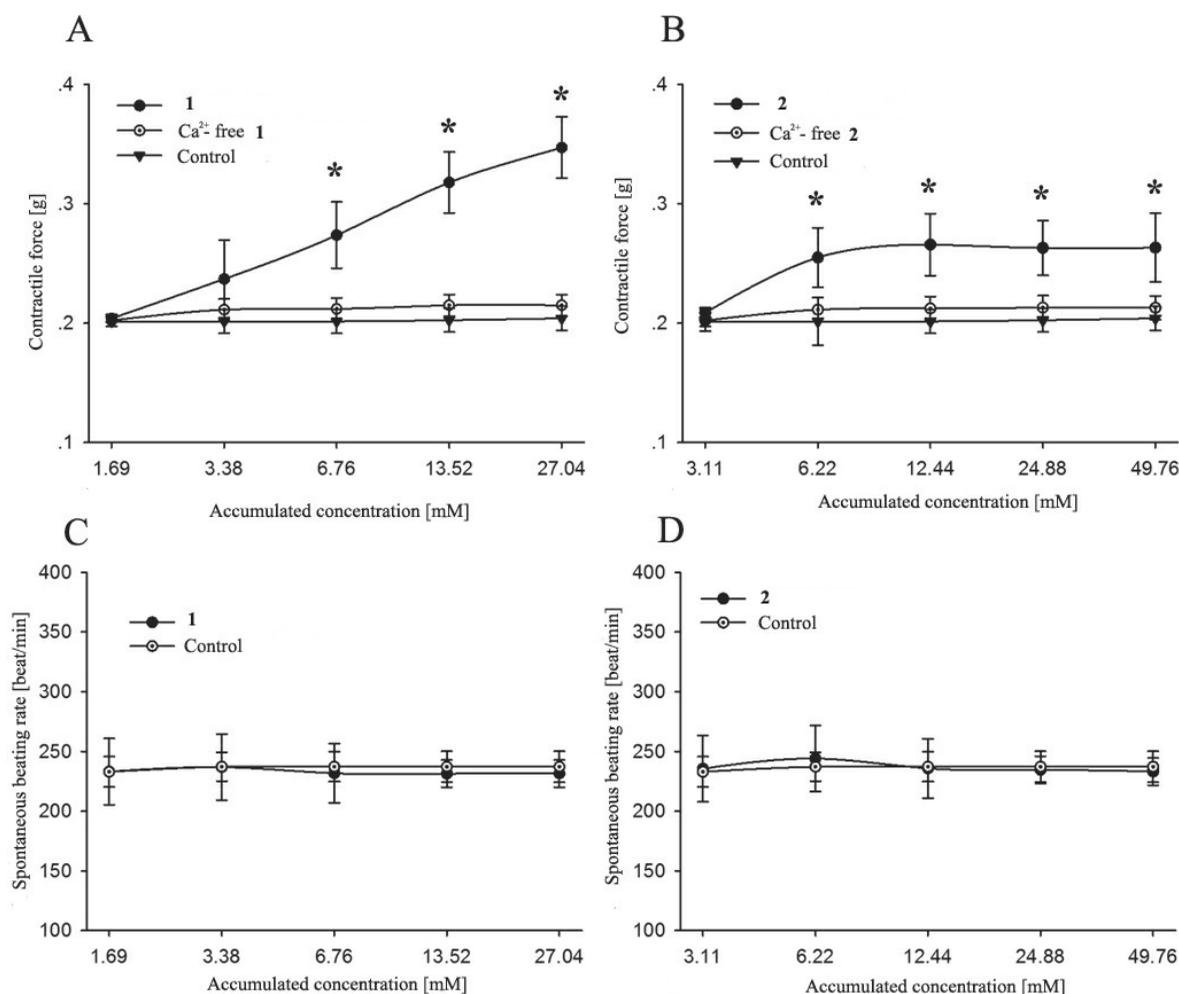


Fig. 3. Effects of **1** and **2** on contractile force and spontaneous beating rate of the right atrium. (A, B) Effect of **1** (A) and **2** (B) on the contractile force of the right atrium as function of its concentration. (C, D) Cumulative effect of **1** (C) and **2** (D) on the spontaneous beating rate. Data are expressed as mean \pm SD, $n = 6$; * $p < 0.05$ versus control group.

inotropic effect on the myocardium, since Ca^{2+} plays a crucial role in the excitation-contraction coupling in cardiac myocytes. Further investigations at the organ level confirmed this assumption when **1** and **2** were added to the organ bath cumulatively. Fig. 3A indicates that **1** increased the contractile force of the right atrium in a dose-dependent manner, with no saturation at 27 mM (0.35 g contractile force). Due to limitations in the availability of the sample, higher concentrations were not tested. These inotropic effects were abolished by pretreating the myocardium in Ca^{2+} -free solution.

The inotropic effect of **2** was lower and was already saturated at 6.22 mM (Fig. 3B). The spontaneous beating rate of the right atrium [(235 ± 36) beats/min] did not change significantly in the presence of **1** (Fig. 3C) or **2** (Fig. 3D). Positive ino-

tropic effects of **1** and **2** on the papillary muscle of the right ventricle were also observed in this experiment, but to the limited sample amounts available, these effects were not studied in detail (data not shown).

To the best of our knowledge, the lactam compounds **1**–**3** have been isolated from *Tricholoma matsutake* (S. Ito & Imai) Sing. for the first time. Compounds **1** and **2** exerted positive inotropic effects on isolated myocardial tissue. (For lack of material, compound **3** could not be tested in a comparable way.) We propose that these two lactam compounds could be candidates as inotropic agents. Their *in vivo* evaluation and the elucidation of their mechanism(s) of action require further studies, for which larger amounts of the compounds must be obtained, possibly by chemical synthesis.

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