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Graphical abstract

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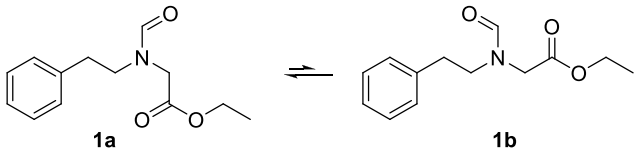

Leccinine A, an endoplasmic reticulum stress suppressive compound from the edible mushroom *Leccinum extremiorientale*

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ABSTRACT

Leccinine A (**1**) along with a known compound (**2**), were isolated from the edible mushroom *Leccinum extremiorientale*. The structure of **1** was determined by the interpretation of spectral data. Leccinine A showed protective activity against endoplasmic reticulum stress-dependent cell death. Seven analogues (**3** to **9**) of **1** were synthesized in order to evaluate the structure-activity relationship, and the result indicated that the formamide group of **1** was indispensable for the activity.

1. Introduction

The endoplasmic reticulum (ER) is the organelle responsible for folding and modification of proteins destined for the secretory pathway and endosomal compartment. ER stress-dependent neuronal death has been reported to cause some neurodegenerative diseases, such as Alzheimer, Parkinson, and Huntington diseases,[1-3] and amyloid-beta (Aβ) peptides was reported to induce neuronal cell death via ER stress signaling.[4] ER stress signals are induced when functions of the ER are impaired by various physiological and pathological conditions. ER stress has been reported to cause not only neurodegenerative diseases but also some other diseases, such as diabetes, atherosclerosis, heart and liver disease.[5] Therefore, the protective activity against ER stress is an important target for the cure or prevention of these diseases. Recently, beneficial effects of natural products that protect cell death by ER stress have been reported. For example, we have reported the isolation of dilinoleoylphosphatidylethanolamine and 3-hydroxyhericenone F as ER stress-suppressive compounds from the mushroom *Hericium erinaceum*.[6,7] Three furanones and a phenylpentanone from the mushroom *Mycoleptodonoides aitchisonii*,[8] and termitomycamides A to E from the mushroom *Termitomyces titanicus* also have

been reported as the suppressive compounds by us.[9] In the course of our continuing search for ER stress protecting compounds from mushrooms, we found a novel active compound from the edible mushroom *Leccinum extremiorientale*. This mushroom, having a red brown areolate cap, grows worldwide, but distributed mainly in the northern temperate zone. Here we describe the isolation and structural determination of the active compound, and structure-activity relationship by comparing the activity of the compound with those of its seven synthesized analogues.

2. Results and discussion

The fresh fruiting bodies of *L. extremiorientale* were extracted with EtOH and acetone, successively. The combined extract of the mushroom was divided into a hexane soluble-, an EtOAc soluble- and a water soluble-fractions. The hexane soluble- and the EtOAc soluble-fractions were repeatedly subjected to column chromatography, followed by HPLC to afford compounds **1** and **2**, respectively.

Leccinine A (**1**) was purified as a colorless oil. Its molecular formula was determined as $C_{13}H_{17}NO_3$ by HRESIMS [*m/z* 258.1077 $[M + Na]^+$ (calcd for $C_{13}H_{17}NaNO_3$, 258.1107)]. The NMR data revealed that most of the proton and carbon

signals were paired to each other. The MS and the NMR data implied that compound **1** was a set of rotational isomers. The ratio of the main isomer to the minor one was determined as 3 to 1 by their integral values in the ^{1}H NMR spectroscopy. The structure of the major isomer (**1a**) was elucidated by interpretation of NMR spectra including DEPT, COSY, HMBC, and HMQC (Fig. 1). The complete assignment of the protons and the carbons was accomplished as shown in Table 1. The presence of the phenylethyl moiety was suggested by the COSY correlations (bold line in Fig. 1) and the HMBC correlations (H-1′/C-1″, H-1′/C-2′, H-2′/C-1′, H-2′/C-1″, H-2′/C-2″,-6″, H-2″,-6″/C-2′, H-2″,-6″/C-3″,-5″, H-2″,-6″/C-4″, H-3″,-5″/C-1″, H-3″,-5″/C-2″,-6″, H-4″/C-2″,-6″ H-4″/C-3″,-5″, H-5″/C-3″). The ethoxycarbonylmethyl was elucidated by the COSY (bold line in Fig. 1) and the HMBC correlations (H-1‴/C-1, H-1‴/C-2‴, H-2‴/C-1‴, H-2/C-1) and the chemical shift of C-1 (δ_C 170.2). The other part, N-CHO group, was suggested by the molecular formula and the NMR signals at δ_H 7.85 (1H, s) and δ_C 165.6. The connection of the phenylethyl, the ethoxycarbonylmethyl and the N-CHO moieties was determined by the HMBC correlations (N-CHO/C-2, N-CHO/C-1′, H-2/N-CHO, H-2/C-1′, H-1′/C-2, H-1′/N-CHO). The structure of the minor isomer (**1b**) was also deduced by the interpretation of spectroscopic data. All the data allowed us to conclude that **1** was ethyl 2-(*N*-phenethylformamido)acetate.

Detailed assignments of the NMR signals revealed that the differences of the chemical shifts between the two isomers were larger near the foramide (N-CHO) than the other parts (Table 1). The significant NOEs were observed between the formyl proton at δ_H 7.85 and H-1′ (δ_H 3.63, t, 2H) in **1a**, and the proton at δ_H 8.02 and H-2 (δ_H 4.03, s, 2H) in **1b** in the NOE difference experiment, indicating that the structures of the two rotational isomers are as shown. These rotational isomers, **1a** and **1b**, appeared as two different signals from each other in reverse-phase HPLC and were able to be separated. However, after the isolation, each isomer also gave a set of two signals of the rotational isomers at the same molar ratio as that before the isolation in HPLC analysis (data not shown). In addition, the formyl analogues (**3**, **7** and **9**, as described later) of **1** also existed as a set of rotational isomers. Similar isomers have been reported for tertiary amide-containing compounds such as rhizopodin and haliclonin A.[10,11]

Compound **2** was identified as pyrrolezanthine, which had been isolated from a plant *Zanthoxylum simulans*, by analyses of NMR and mass spectra.[12] To our knowledge, this compound is the first isolation from fungi including mushrooms.

Leccinine A (**1**) was subjected to the protective activity assay against ER stress-dependent cell death caused by tunicamycin (TM) or thapsigargin (TG). ER stress was induced by the addition of TM or TG into the culture medium of Neuro2a cells in

the presence or absence of **1**. TM is an inhibitor of *N*-linked glycosylation and the formation of *N*-glycosidic protein-carbohydrate linkages.[13] It specifically inhibits dolichol pyrophosphate-mediated glycosylation of asparaginyl residues of glycoproteins and induces the ER stress.[14] TG, an inhibitor of the sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase, also induces ER stress by disrupting the homeostatic balance of the Ca^{2+} concentration in the ER.[15] Leccinine A (**1**) showed the significant protective activity against TG-toxicity dose-dependently, although it did not show the activity in the test using TM (Fig. 2).

To investigate structure-activity relationship of **1** on protective activity against ER stress-dependent cell death, analogues (**3-9)** of **1** were synthesized as described previously except for a new compound (**7**).[16-21] Compounds **4** and **6-8** did not show any activity. Compounds **3**, **5** and **9** having a formamide group in each molecule exhibited the tendency of protective activity in a dose-dependent manner as **1**, although the data showed no significant differences (data not shown). These results indicated that the backbone carbon chain requires the formamide group of **1** and is indispensable for the activity.

3. Experimental

3.1. General

^{1}H NMR spectra (one- and two-dimensional) were recorded on a JEOL lambda-500 spectrometer at 500 MHz and 270 MHz, while ^{13}C NMR spectra were recorded on the same instrument at 125 MHz. The HRESIMS spectra were measured on a JMS-T100LC mass spectrometer. A JASCO grating infrared spectrophotometer was used to record the IR spectra. HPLC separations were performed with a JASCO Gulliver system using reverse-phase HPLC columns (Capcell pak C18 AQ, Shiseido, Japan; COSMOSIL Cholester Waters, Nacalai tesque, Inc. Japan; Develosil C30-UG-5, Nomura chemical Co., Ltd, Japan; Wakosil-2 5C18 HG Prep, Wako, Japan; XBridge Prep Phenyl ODB, Waters, Japan). Silica gel plate (Merck F_{254}) and silica gel 60 N (Merck 100-200 mesh) were used for analytical TLC and for flash column chromatography, respectively.

3.2. Fungus materials

Mature fruiting bodies of *L. extremiorientale* were collected at Narusawa village, Yamanashi Prefecture in Japan, in August 2007.

3.3. Extraction and Isolation

The fresh fruiting bodies of *L. extremiorientale* (14.9 kg) were extracted with EtOH (20 L, three times) and then acetone (10 L). The combined solution was evaporated under reduced pressure and the concentrate was partitioned between hexane and H_2O and then between EtOAc and H_2O. The EtOAc-soluble part (32.4 g) was fractionated by silica gel flash column chromatography (CH_2Cl_2/EtOAc 7:3, 1:1; EtOAc; EtOAc /MeOH 4:1; and MeOH, 2.0 L each) to obtain eleven fractions (fractions 1 to 11), and fraction 8 (3.0 g) was further separated by silica gel flash column chromatography (CH_2Cl_2; CH_2Cl_2/EtOAc 95:5, 9:1, 6:4, 1:4; EtOAc; MeOH, 1.2 L each), affording fifteen fractions (fractions 8-1 to 8-15). Fraction 8-9 (77.4 mg) was further separated by reverse-phase HPLC (Develosil C30-UG-5, 40% MeOH) to obtain four fractions (fractions 8-9-1 to 8-9-4). Compound **1** (1.0 mg) was obtained from fraction 8-9-3 (5.9 mg) by reverse-phase HPLC (Capcell pak C18 AQ, 50% MeOH). The hexane-soluble part (58.0 g) was fractionated by silica gel flash column chromatography (hexane/CH_2Cl_2 1:1, 1:4; CH_2Cl_2/acetone 9:1, 1:1; acetone; and MeOH, 2.0 L each) to obtain fourteen fractions (fraction 1 to 14), and fraction 5 (27.2 g) was further separated by silica gel flash column chromatography (CH_2Cl_2; CH_2Cl_2/acetone 19:1, 9:1, 7:3, 1:1; and acetone, 1.2 L each) affording thirteen fractions (fraction 5-1 to

5-13). Fraction 5-11 (1.1 g) was further separated by reverse-phase HPLC (Wakosil-2 5C18 HG, 90% MeOH) to obtain nineteen fractions (fraction 5-11-1 to 5-11-19). Compound **2** (0.7 mg) was obtained from fraction 5-11-3 by reverse-phase HPLC (COSMOSIL Cholester Waters, 30% MeOH).

*3.3.1. Leccinine (**1**).* Colorless oil; IR (neat): 2937, 1747, 1675 cm^{-1}; ^{1}H and ^{13}C NMR, see Table 1; ESIMS *m/z* 258 $[M+Na]^+$; HRESIMS *m/z* 258.1077 $[M+Na]^+$ (calcd for $C_{13}H_{17}NaNO_3$, 258.1107).

*3.3.2. Compound **2**.* ^{1}H NMR ($CDCl_3$, 500 MHz) δ 9.56 (1H, s), δ 6.94 (2H, d, 8.0), δ 6.90 (1H, d, 4.0), δ 6.71 (2H, d, 6.5), δ 6.15 (1H, d, 4.0), δ 4.68 (1H, br s), δ 4.49 (2H, t, 7.0), δ 4.31 (1H, s), δ 2.96 (2H, t, 6.5); ESIMS *m/z* 268 $[M+Na]^+$; HRESIMS *m/z* 268.0922 $[M+Na]^+$ (calcd for $C_{14}H_{15}NNaO_3$, 268.0950).

3.4. Synthesis

*3.4.1. N-Phenethylformamide (**3**).* 2-Phenethylamine (1 mmol, 125 μL) was dissolved in pyridine (0.5 mL) and a mixture of formic acid (4 mmol, 150 μL) and diisopropylcarbodiimide (DIPCD, 4 mmol, 600 μL) was added to the solution. The resulting mixture was stirred for 72 h at room temperature. The products were purified

by silica gel flash column chromatography (CH_2Cl_2; CH_2Cl_2/acetone 9:1, 5:5; acetone; acetone/MeOH 5:5) and then reversed-phase HPLC (XBridge Prep Phenyl OBD, 16% MeOH) to give **3** (22.7 mg, 15.0% yield). Compound **3** was obtained a set of rotational isomers (**3a** and **3b**). The molar ratio of **3a** to **3b** was 5.7 to 1. **3a**: ^{1}H NMR ($CDCl_3$, 270 MHz) δ 8.03 (1H, s), δ 7.07-7.27 (5H, m), δ 5.61 (1H, br, s), δ 3.50 (2H, q, 7.2), δ 2.76 (2H, t, 7.0). **3b**: δ 7.83 (1H, d, 11.9), δ 7.07-7.27 (5H, m), δ 3.39 (2H, q, 6.8), δ 2.76 (2H, t, 7.0). ESIMS *m/z* 172 $[M+Na]^+$; HRESIMS *m/z* 172.0738 $[M+Na]^+$ (calcd for $C_9H_{11}N_1NaO_1$, 172.0746).[16]

3.4.2. N-Phenethylacetamide (4). 2-Phenethylamine (1 mmol, 125 μL) was dissolved in pyridine (150 μL) and a mixture of acetyl chloride (1 mmol, 70 μL) and diethylamine (0.1 mmol, 10 μL) was added to the solution. The resulting mixture was stirred for 72 h at room temperature. The products were purified by silica gel flash column chromatography (CH_2Cl_2; CH_2Cl_2/acetone 9:1, 5:5; acetone; acetone/MeOH 5:5) and then reversed-phase HPLC (XBridge Prep Phenyl OBD, 70% MeOH) to give **4** (16.0 mg, 9.8% yield). **4**: ^{1}H NMR ($CDCl_3$, 270 MHz) δ 7.16-7.33 (5H, m), δ 3.50 (2H, d, 6.8), δ 2.79 (2H, t, 7.0), δ 1.92 (3H, s); ESIMS *m/z* 186 $[M+Na]^+$; HRESIMS *m/z* 186.0888 $[M+Na]^+$ (calcd for $C_{10}H_{13}N_1NaO_1$, 186.0895).[17]

*3.4.3. Ethyl formamidoacetate (**5**).* Trimethyl orthoformate (10 mmol, 1.1 mL) and glycine ethyl ester hydrochloride (10 mmol, 1.39 g) were stirred at 110˚C for 2 h. The products were purified by reversed-phase HPLC (Capcell pak C18 AQ, 10% MeOH) to afford **5** (126 mg, 9.6% yield). **5**: ^{1}H NMR ($CDCl_3$, 270 MHz) δ 8.16 (1H, s), δ 4.14 (2H, q, 7.0), δ 3.98 (2H, d, 5.4), δ 1.12 (3H, t, 7.3); ESIMS *m/z* 285 $[2M+Na]^+$; HRESIMS *m/z* 285.1071 $[2M+Na]^+$ (calcd for $C_{10}H_{18}N_2NaO_6$, 285.1063).[18]

*3.4.4. Ethyl 2-(phenethylamino)acetate (**6**).* 2-Phenethylamine (6 mmol, 755 μL) was dissolved in pyridine (500 μL) and a mixture of ethyl bromoacetate (6 mmol, 660 μL) and diethylamine (0.6 mmol, 60 μL) was added to the solution. The resulting mixture was stirred for 24 h at room temperature. The products were purified by silica gel flash column chromatography (acetone) and then reversed-phase HPLC (Capcell pak C18 AQ, 50% MeOH) to obtain **6** (45.4 mg, 3.6% yield). **6**: ^{1}H NMR ($CDCl_3$, 270 MHz) δ 7.12-7.29 (5H, m), δ 4.14 (2H, q, 7.0), δ 3.31 (2H, s), δ 2.87 (2H, t, 2.7), δ 2.77 (2H, t, 2.7), δ 1.23 (3H, t, 7.0); ESIMS *m/z* 208 $[M+H]^+$; HRESIMS *m/z* 208.1338 $[M+H]^+$ (calcd for $C_{12}H_{19}N_1O_2$, 208.1359).[19]

3.4.5. Ethyl 2-(N-phenethylacetamido)acetate (7). **6** (100 mmol, 20 mg) was dissolved in pyridine (500 μL) and a mixture of acetyl chloride (0.4 mmol, 30 μL) and DIPCD (0.4 mmol, 60 μL) was added to the solution. The resulting mixture was stirred for 24 h at room temperature. The products were purified by silica gel flash column chromatography (CH_2Cl_2; CH_2Cl_2/acetone 9:1, 5:5; acetone; acetone/MeOH 5:5) and then reversed-phase HPLC (Capcell pak C18 AQ, 50% MeOH) to give **7** (4.7 mg, 19% yield). Compound **7** was obtained a set of rotational isomers (**7a** and **7b**). The molar ratio of **7a** to **7b** was 2.5 to 1. **7a**: ^{1}H NMR (CD_3OD, 270 MHz) δ 7.19-7.32 (5H, m, H-2″, H-3″, H-4″, H-5″, H-6″), δ 4.18 (2H, q, 7.0, H-1‴), δ 4.04 (2H, s, H-2), δ 3.63 (2H, t, 7.2, H-1′), δ 2.88 (2H, t, 7.1, H-2′), δ 1.86 (3H, s, NCO-*Me*), δ 1.26 (3H, t, 7.0, H-2‴). **7b**: δ 7.19-7.32 (5H, m, H-2″, H-3″, H-4″, H-5″, H-6″), δ 4.18 (2H, q, 7.0, H-1‴), δ 4.09 (2H, s, H-2), δ 3.55 (2H, t, 7.7, H-1′), δ 2.80 (2H, t, 7.6, H-2′), δ 2.01 (3H, s, NCO-*Me*), δ 1.26 (3H, t, 7.0, H-2‴). ESIMS *m/z* 272 $[M+Na]^+$; HRESIMS *m/z* 272.1254 $[M+Na]^+$ (calcd for $C_{14}H_{19}N_1NaO_3$, 272.1262).

*3.4.6. 2-(Phenethylamino)acetic acid (**8**).* Bromoacetic acid (1 mmol, 139 mg) was dissolved in pyridine (500 μL) and a mixture of 2-phenethylamine (1 mmol, 125 μL) and diethylamine (0.1 mmol, 10 μL) was added to the solution. The resulting mixture

was stirred for 48 h at room temperature. The products were purified by silica gel flash column chromatography (CH_2Cl_2; CH_2Cl_2/acetone 9:1, 5:5; acetone; acetone/MeOH 5:5) and then reversed-phase HPLC (Capcell pak C18 AQ, 33% MeOH) to obtain **8** (23.4 mg, 13% yield). **8**: ^{1}H NMR (CD_3OD, 270 MHz) δ 7.21-7.35 (5H, m), δ 3.50 (2H, s), δ 3.25 (2H, q, 7.0), δ 2.97 (2H, t, 7.0); ESIMS *m/z* 381 $[2M+Na]^+$; HRESIMS *m/z* 381.1782 $[2M+Na]^+$ (calcd for $C_{20}H_{26}N_2NaO_4$, 381.1790).[20]

*3.4.7. 2-(N-Phenethylformamido)acetic acid (**9**).* Glyoxylic acid monohydrate (10 mmol, 1.84 g) was dissolved in distilled water (5 mL) and 2-phenethylamine (5 mmol, 630 μL) was added to the solution. The resulting mixture was stirred for 24 h at room temperature. The products were purified by silica gel flash column chromatography (acetone; acetone/MeOH 5:5) and then reversed-phase HPLC (Capcell pak C18 AQ, 28% MeOH) to afford **9** (113 mg, 11% yield). Compound **9** was obtained a set of rotational isomers (**9a** and **9b**). The molar ratio of **9a** to **9b** was 2.8 to 1. **9a**: ^{1}H NMR (CD_3OD, 270 MHz) δ 7.83 (1H, s), δ 7.20-7.28 (5H, m), δ 4.06 (2H, s), δ 3.61 (2H, t, 7.0), δ 2.86 (2H, t, 7.0). **9b**: δ 8.02 (1H, s), δ 7.20-7.28 (5H, m), δ 3.99 (2H, s), δ 3.56 (2H, t, 8.1), δ 2.86 (2H, t, 7.0). ESIMS *m/z* 230 $[M+Na]^+$; HRESIMS *m/z* 230.0792 $[M+Na]^+$ (calcd for $C_{11}H_{13}N_1NaO_3$, 230.0794).[21]

*3.4.8. Ethyl 2-(N-phenethylformamido)acetate (**1**).* **6** (100 mmol, 20 mg) was dissolved in pyridine (500 μL) and a mixture of formic acid (0.4 mmol, 15 μL) and DIPCD (0.4 mmol, 60 μL) was added to the solution. The resulting mixture was stirred for 24 h at room temperature. The products were purified by silica gel flash column chromatography (CH_2Cl_2; CH_2Cl_2/acetone 9:1, 5:5; acetone; acetone/MeOH 5:5) and then reverse-phase HPLC (Capcell pak C18 AQ, 50% MeOH) to give **1** (7.7 mg, 33%); ^{1}H and ^{13}C NMR, see Table 1.

3.5. Bioassay

Neuro2a cells were obtained from the Health Science Research Resources Bank, Japan, and maintained in the Dulbecco‘s modified Eagles medium (D-MEM) (SIGMA, USA) supplemented with 10% (v/v) fetal bovine serum (FBS), unless particularly noted. Cell viability analysis was performed by 3-(4,5-dimethyl-2-thiazolyl)2,5-diphenyl-2H-tetrazolium bromide (MTT) assay. MTT assay was performed as follows; Neuro2a cells were cultured in 96-well plates at cell density 5000 cells/well and after one-day cultivation, the cells were cultured in D-MEM without FBS, and with 0.5 μg/mL of tunicamycin (or 20 nM thapsigargin) and varying

concentrations of test compounds were applied to the medium. The cells were incubated for 24 h, and then the viability was measured by MTT assay, as described previously.[22] Briefly, 0.25 mg/mL of MTT in D-MEM without FBS were added onto the cells and incubated for 2 h. The incubation was terminated by addition of 20% SDS (v/w) and 50% DMSO (v/v) in water. The absorbance at 570 nm of the reaction mixture was measured by a microplate reader (Molecular Devices, USA).

3.6. Statistical Analysis

Data collected were analyzed statistically using Tukey-Kramer multiple comparisons tests to determine significant difference in the data among the groups. *P* values less than 0.01 were considered significant. The values are expressed as mean ± SEM.

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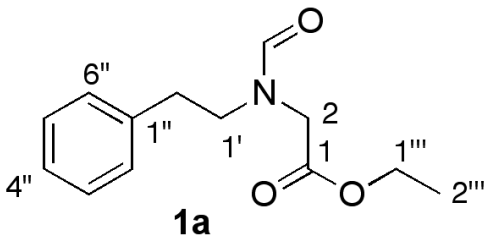
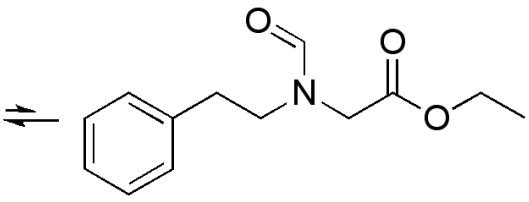
Figure Legends

Figure 1. COSY and HMBC correlations of **1a**.

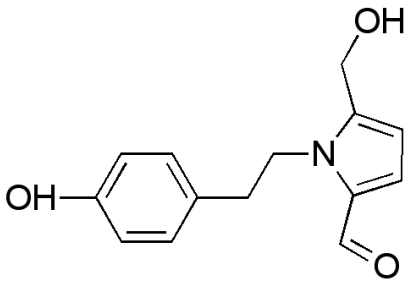
Figure 2. Protective activity of **1** against ER stress-dependent cell death. Neuro2a cells were incubated with various concentrations of leccinine A (**1**) in the absence (A) or presence of 0.5 μg/mL of tunicamycin (B) or 20 nM thapsigargin (C) for 24 h. The cell viabilities were analyzed by MTT assay, and the values were represented as the mean ± SEM of the relative percentage of surviving cells compared with the untreated cells ($n = 16$). (∗) $p < 0.01$, Tukey-Kramer multiple comparisons tests.



1a



1b



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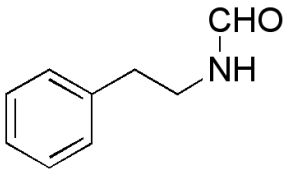

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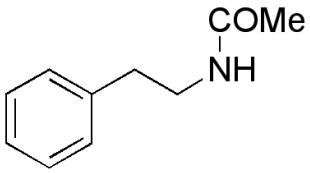

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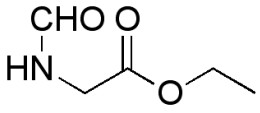

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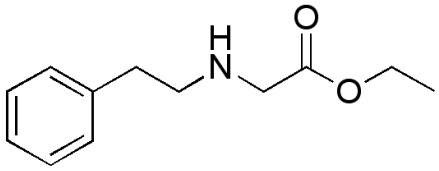

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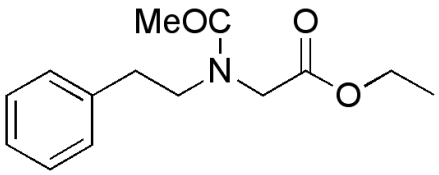

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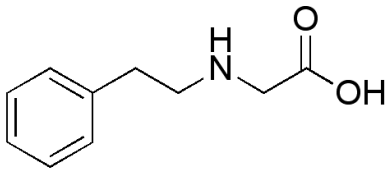

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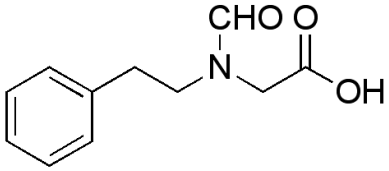

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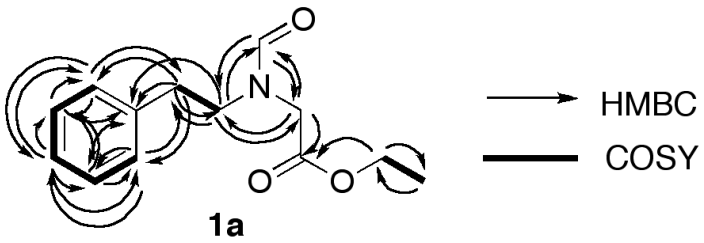

1a

Choi et al., Fig.1

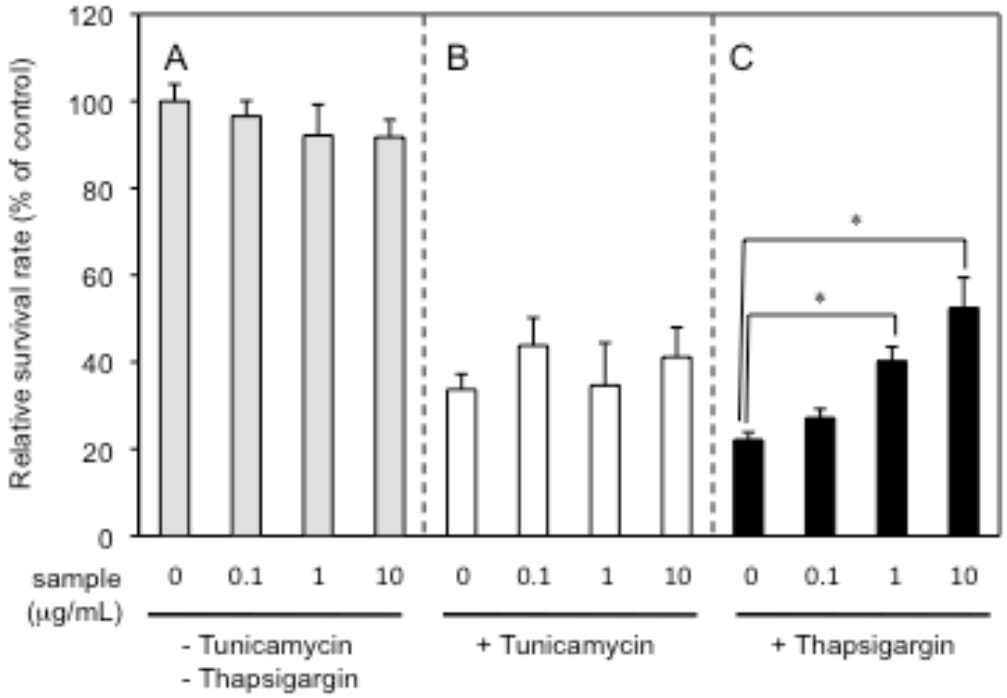

Fig 2. Choi et al.

Table 1. NMR Spectroscopic Data for Leccinine A (**1**)[a]

position	δ_H (mult, *J* in Hz)	δ_C	HMBC
1[b]		170.2	
1[c]		171.1	
2[b]	4.08 (s)	45.5	N-CHO, C-1, 1′
2[c]	4.03 (s)	50.3	
1′[b]	3.63 (t, 7.0)	51.3	N-CHO, C-2, 2′, 1″
1′[c]	3.55 (t, 7.6)	46.9	
2′[b]	2.87 (t, 7.0)	35.8	C-1′, 1″, 2″, 6″
2′[c]	2.82 (t, 7.6)	34.4	
1″[b]		139.6	
1″[c]		140.0	
2″	7.21 (d, 9.0)	130.1	C-2′, 3″, 4″
3″	7.28 (m)	129.7	C-1″, 2″
4″	7.21 (m)	127.7	C-2″, 223″, 6″
5″	7.28 (m)	129.7	C-1″, 3″, 6″
6″	7.21 (d, 9.0)	130.1	2′, 4″
1‴	4.19 (q, 7.0)	62.4	C-1, 2‴
2‴	1.26 (t, 7.0)	14.4	C-1‴
N-CHO[b]	7.85 (s)	165.6	C-2, 1′
N-CHO[c]	8.02 (s)	166.1	

[a] Data were obtained in CD_3OD (500 MHz).
[b,c] Denote chemical shifts for the major (**1a**) and minor rotational isomer (**1b**), respectively.