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Note

Isolation of Bioactive Steroids from the *Stropharia rugosoannulata* Mushroom and Absolute Configuration of Strophasterol B

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The absolute configuration of strophasterol B (1) isolated from the fruiting bodies of *Stropharia rugosoannulata* was determined by an X-ray crystallographic analysis. Three compounds (2 to 4) were isolated from the mushroom which suppressed or promoted lettuce growth.

Key words: *Stropharia rugosoannulata*; mushroom; absolute configuration; strophasterol B; lettuce growth assay

The *Stropharia rugosoannulata* mushroom is called saketsubatake in Japanese, and wine-cap stropharia in English. It belongs to the Strophariaceae family which is widespread in northern temperate zones throughout the world. It is edible and is cultivated for food. We have reported in the previous papers the isolation of osteoclast formation-suppressing compounds, anti-fungal ones, and endoplasmic reticulum (ER) stress-suppressing ones.[1,2] Among them, strophasterols A to D had the same unprecedented steroid skeleton, although the stereochemistry of the steroids has not yet been determined except for strophasterol A.[2] We describe here the determination of the absolute configuration of strophasterol B (**1**) and the further isolation of bioactive steroids from the mushroom.

Strophasterol B (**1**) has been isolated from the mushroom, but its stereochemistry was not determined in the previous study. Its relative stereochemistry was determined in this study by an X-ray crystallographic analysis (Fig. 1A). The CD data (λext ($\Delta\varepsilon$): 211 (+7.8), 232 (−0.5), 244 (+1.7), 339 (−1.6) nm) for **1** are similar to those (211 (+8.8), 234 (+1.6), 245 (+3.7), 341 (−1.2) nm) for strophasterol A whose absolute configuration had been determined by an X-ray crystallographic analysis of its bis(*p*-bromo)benzoate (Fig. 1B).[2] These results lead to the conclusion shown in Scheme 1 for the absolute configuration of **1**.

Fresh fruiting bodies of *S. rugosoannulata* were extracted with EtOH and then with acetone. After the extracts had been combined and concentrated, the resulting mixture was partitioned between hexane and H_2O, $CHCl_3$ and H_2O, and then EtOAc and H_2O. The hexane-soluble part was fractionated by repeated chromatography, and three compounds (**2** to **4**) were purified. These compounds have been previously reported, and were identified by comparing their spectroscopic data with those previously reported. Compound **2** has been isolated from the mushrooms of *Amanita pantherina*, *Amanita virgineoides*, *Lactarius piperatus*, *Lyophyllum shimeji*, *Tricholoma portentosum*, *Hypsizigus marmoreus* and *Lentinula edodes*.[3] Compounds **3** and **4** have been isolated from the fruiting bodies of *Tricholoma portentosum*[3] and *Grifola frondosa*,[4] and from the *Paecilomyces* sp. fungus.[5] Compound **4** showed the moderate cytotoxicity toward five tumor cell lines.[5]

The effect of compounds **2** to **4** on lettuce growth was exmined. Compounds **3** and **4** inhibited the hypocotyl growth of lettuce at 1 μmol/paper, 100 nmol/paper, 10 nmol/paper and 1 nmol/paper (respective length of growth compared with the control ± standard deviation: **3**, 60.8 ± 19.2%, 72.2 ± 12.9%, 78.4 ± 15.4%, 73.2 ± 6.8%; **4**, 64.9 ± 6.4%, 67.0 ± 8.1%, 68.0 ± 8.3%, 79.4 ± 9.1%) with statistically significant difference. In addition, **3** and **4** promoted the root growth of lettuce at 100 nmol/paper, 10 nmol/paper and 1 nmol/paper (**3**, 122.4 ± 20.1%, 118.2 ± 8.1%, 123.8 ± 15.1%, respectively), and at 10 nmol/paper and 1 nmol/paper (**4**, 124.5 ± 16.2%, 143.4 ± 8.3%, respectively) with statistically significant difference. Compound **2** exhibited no activity. A comparison of the structures among **2**, **3** and **4** indicates that the double bond between C-8 and C-9 or C-8 and C-14 in the sterol skeleton was necessary for regulating the lettuce growth.

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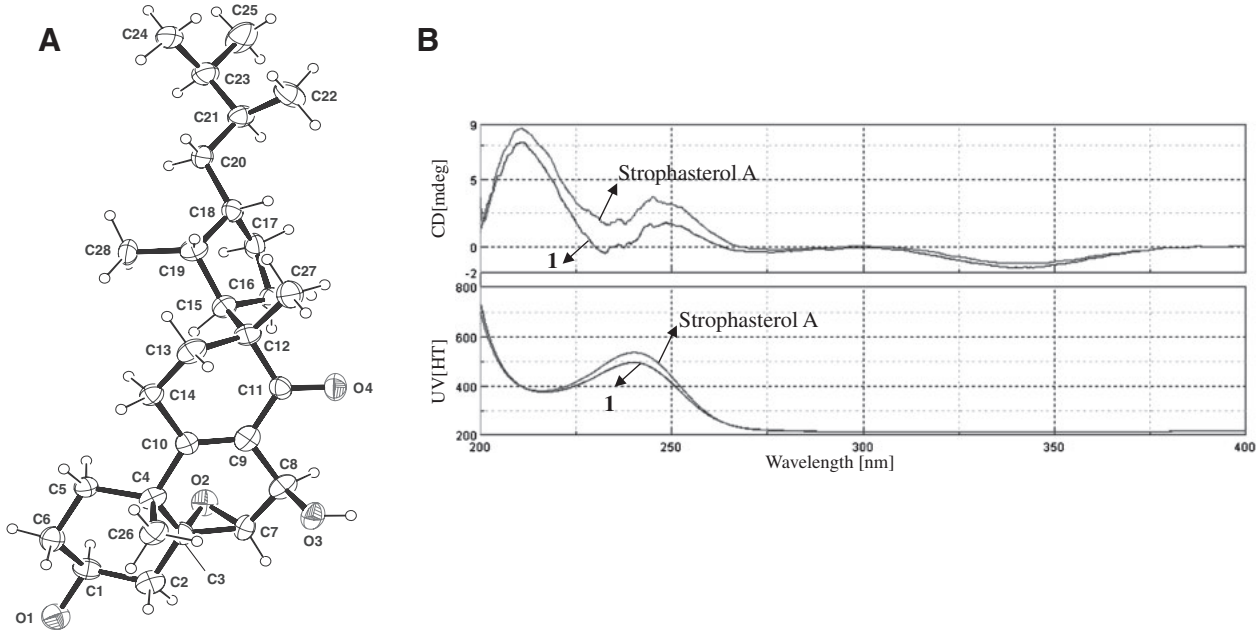
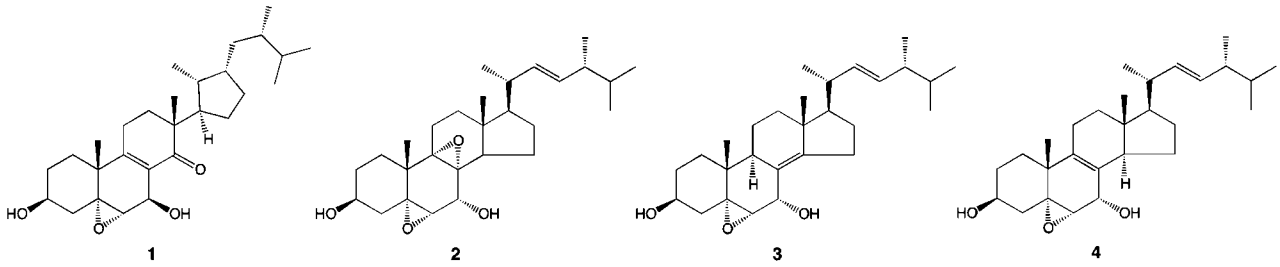


Fig. 1. Stereochemistry of **1**.

A, ORTEP drawing of **1** with ellipsoids at the 30% probability level. Hydrogen atoms are shown as small spheres of arbitrary radii. B, CD spectra of **1** and strophasterol A.



Scheme 1.

Experimental

General experiments. ^{1}H-NMR spectra (one- and two-dimensional) were recorded by a Lambda-500 spectrometer at 500 MHz, and ^{13}C-NMR spectra were recorded by the same instrument at 125 MHz (Jeol, Tokyo, Japan). ESIMS data were measured by a JMS-T100LC mass spectrometer (Jeol, Tokyo, Japan). Specific rotation values were measured with a Jasco DIP-1000 polarimeter. HPLC separation was performed with a Jasco Gulliver system, using reverse-phase HPLC columns (Wakosil-II 5C18 HG Hrep, Wako, Osaka, Japan; COSMOSIL 5PYE waters, Nacalai Tesque, Kyoto, Japan). Silica gel plates (Merck F_{254}) and silica gel 60N (Merck 100–200 mesh) were respectively used for analytical TLC and for flash column chromatography (Merck, Darmstadt, Germany).

Fungal strain and culture conditions. The strain of *S. rugosoannulata* was isolated from a fruiting body collected in Gunma Prefecture, Japan, in August 2000. Voucher material has been deposited in Gunma Prefectural Forestry Experiment Station, Japan. The culture medium was prepared by mixing ground flakes of Japanese oak and fresh rice bran at a weight ratio of 8:2 adjusted to 65% moisture. The medium (2.5 kg) was packed in polypropylene bags and autoclaved. Precultured mycelia were inoculated into each bag of the medium. After cultivating for 5 months in the dark at 25 °C and 65% humidity, each set of two bags was transplanted into a plastic planter filled with bark compost. Fruiting body induction and further cultivation were done in a greenhouse without controlling the temperature, humidity, or light for about 2 months.

Extraction and isolation. Fresh fruiting bodies of *S. rugosoannulata* (20.0 kg) were extracted three times with EtOH (42 L) and then three times with acetone (20 L). After the solutions had been combined and concentrated under reduced pressure, the concentrate was partitioned first between hexane and H_2O, second with $CHCl_3$ and H_2O, and third with EtOAc and H_2O. The hexane-soluble part (109.5 g) was fractionated by silica gel flash column chromatography (hexane; 70%, 50%, 20% hexane/CH_2Cl_2; CH_2Cl_2; 90%, 80%, 70%, 40% CH_2Cl_2/EtOAc; EtOAc; 80%, 70%, 50%, 30% EtOAc/MeOH; and MeOH; 1.5 L each) to obtain 28 fractions (fractions 1 to 28). Fraction 17 (5.004 g) was further separated by silica gel flash column chromatography (CH_2Cl_2; 90%, 80%, 70%, 60%, 50%, 40%, 30%, 20%, 10% CH_2Cl_2/EtOAc; 90%, 80%, 70%, 60%, 40%, 30%, 20% EtOAc/MeOH; and MeOH; 0.5 L each) to give 17 fractions (fractions 17-1 to 17-17), and 19 (1.832 g) was further separated (EtOAc; 90%, 80%, 70%, 60%, 50%, 30%, 20%, 10% EtOAc/MeOH; and MeOH; 0.5 L each) to give 11 fractions (fractions 19-1 to 19-11). Fraction 17-11 (857 mg) was further separated by silica gel flash column chromatography (CH_2Cl_2; 90%, 80%, 70%, 60%, 50%, 30%, 20%, 10% CH_2Cl_2/EtOAc; EtOAc; 90%, 80%, 60% EtOAc/MeOH; and MeOH; 0.2 L each) to give nine fractions (fractions 17-11-1 to 17-11-9). Fraction 17-11-6 (605 mg) was separated by reverse-phase HPLC (Wakosil-II 5C18, 5PYE, 90% MeOH) to afford **3** (34.6 mg) and **4** (21.2 mg). Fraction 19-4 (80.8 mg) was further separated by reverse-phase HPLC (Wakosil-II 5C18, 90% MeOH) to afford **2** (4.5 mg). The isolation of **1** has been previously reported, 1.1 mg of **1** being isolated from 20.0 kg of the fruiting bodies.[2)]

X-Ray crystallographic analysis. Strophasterol B (**1**) was crystallized in CH_2Cl_2. $C_{28}H_{44}O_4 \cdot 1.38(H_2O)$, $M = 469.49$, monoclinic, $a = 19.022(3)$ Å, $b = 6.7868(9)$ Å, $c = 21.685(3)$ Å, $\beta = 109.026(8)^\circ$, $V = 2646.5(6)$ Å^3, $T = 173$ K, $P2_1$, $Z = 4$, $\lambda = 0.68680$ Å, $\mu(\lambda = 0.68680) = 0.073$ mm^{-1}, $F(000) = 1031$. The size of the crystal used for measurements was $0.376 \times 0.047 \times 0.028$ mm. Diffraction data were collected at SPring-8 BL02B1 beamline (Hyogo,

Japan) with a Rigaku Mercury2 CCD detector.[6] 13348 reflections were collected in the range $0.96 < \theta < 24.44$, of which 8082 were unique ($R_{int} = 0.0599$) reflections. The structure was refined by the full-matrix least-squares procedure on F^2 values using all unique reflections. The final R indices were $R(F) = 0.1627$ and $wR(F^2) = 0.3336$ (all reflections) with goodness-of-fit = 1.041. The crystallographic data have been deposited at The Cambridge Crystallographic Data Centre and allocated the deposition number CCDC 925655. The data can be obtained free of charge *via* www.ccdc.cam.ac.uk/products/csd/request.

Bioassay. Lettuce seeds were put on filter paper (Advantec no. 2, ϕ 55 mm; Toyo Roshi Kaisha, Japan) soaked in distilled water in a Petri dish (ϕ 60 × 20 mm) and incubated for 1 d in a growth chamber in the dark at 25 °C. Each sample was dissolved in 1 mL of methanol (1, 10^{-1}, 10^{-2}, and 10^{-3} μmol/mL) and then poured on to filter paper (Advantec no. 2, ϕ 55 mm; Toyo Roshi Kaisha, Japan) in a Petri dish (ϕ 60 × 20 mm). After the sample-loaded paper had been air-dried, 1 mL of distilled water was poured on to the loaded paper or an intact filter paper (control). The pre-incubated lettuce seedlings (n = 9 in each Petri dish) were transferred on to the sample-loaded filter paper or control filter paper, and incubated for 3 d in a growth chamber in the dark at 25 °C. The lengths of the hypocotyl and the root were measured with a ruler.

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