SUPPLEMENTARY MATERIAL

A new eremophilane sesquiterpene from the fungus Xylaria sp. V-27 and inhibition activity against degranulation in RBL-2H3 cells

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Supporting Information:

**Experimental procedure**

**Figure S1.** $^1$H NMR Spectrum of Compound 1 (600MHz, CD$_3$OD)

**Figure S2.** $^{13}$C NMR Spectrum of Compound 1 (125 MHz, CD$_3$OD)

**Figure S3.** DEPT Spectra of Compound 1 (125 MHz, CD$_3$OD)

**Figure S4.** COSY Spectrum of Compound 1 (600 MHz, CD$_3$OD)

**Figure S5.** HMQC Spectrum of Compound 1 (600 MHz, CD$_3$OD)

**Figure S6.** HMBC Spectrum of Compound 1 (600 MHz, CD$_3$OD)

**Figure S7.** $^1$H-$^1$H COSY (bold) and Key HMBC (arrows) correlations observed for 1.

**Figure S8.** NOE correlations observed for 1.
Experimental procedure

General procedure

Optical rotation was measured with a Horiba SEPA-300 polarimeter (HORIBA, Kyoto, Japan). IR spectrum was recorded with a Jasco J-20A (JASCO Cooperation, Tokyo, Japan) and Shimadzu UV mini-1240 (SHIMADZU, Kyoto, Japan) spectrophotometers. The HRESIMS was taken on a JEOL HX110 mass spectrometer (JEOL, Tokyo, Japan). 1D and 2D NMR spectra were recorded on a JEOL ECZ-600 at 600 MHz for $^1$H and 150 MHz for $^{13}$C (JEOL, Tokyo, Japan). The chemical shifts are given in ppm (δ), relative to TMS as internal standard, and coupling constants are in Hz. $^1$H, $^{13}$C, COSY, HMQC and HMBC spectra were recorded using standard Jeol standard pulse sequences. Column chromatography was conducted on silica gel 60 (Kanto Chemical Co., Inc., Japan). TLC was carried out on Merck precoated silica gel plates (silica gel 60 F$_{254}$, 20 x 20 cm, Merck, Darmstadt, Germany), and spots were detected by spraying with 10% vanillin in $\text{H}_2\text{SO}_4$ followed by heating, or by UV irradiation.

Fermentation, Extraction and Isolation

The fungal strain Xylaria sp. V-27 was isolated from a dead branch collected in Yamagata, Japan. This fungus was cultivated on sterile steamed unpolished rice (total 200 g, 20 g / petri dish x 10) at 25°C for four weeks. The moldy unpolished rice was extracted with acetone (1.0 L), and acetone extract was concentrated. The resulting aqueous concentrated was partitioned into n-hexane layer (0.3 L),
EtOAc layer (0.5 L) and aqueous layer (0.3 L). Purifications of eluates were monitored by the characteristic intense blue coloration with 10% vanillin in H$_2$SO$_4$ on TLC plates. The EtOAc layer (2.26 g) was chromatographed on a silica gel column with stepwise elution of n-hexane-EtOAc (100:0-0:100) and EtOAc-MeOH (50:50, 0:100), respectively, to afford fractions 1-1 to 1-13. Fraction 1-5 (0.2 g) was rechromatographed on a silica gel column using the stepwise elution (CHCl$_3$-EtOAc) to afford fractions 2-1 to 2-11. Fractions 2-5 and 2-6 (CHCl$_3$-EtOAc, 40:60, 50:50, 40 mg) were further separated by ODS column chromatography eluted with MeCN-H$_2$O (60:40) to give integric acid (2, 50.0 mg) and 13,13-dimethoxyintegric acid (1, 5.5 mg).

**Growth restoring activity of 1 and 2 against the mutant strain**

Growth restored activity of 1 and 2 against the mutant yeast YNS17 strain. Screening was performed according to previous described method. Each sample was dissolved in MeOH and two-fold dilutions of them were used. Difco® yeast-peptone-dextrose (YPD) broth and YPD agar were purchased from Becton Dickinson Biosciences (Franklin Lakes, NJ, USA). The mutant yeast, YNS17 (MATa zds1::TRP1 erg3::HIS3 pdr1::hisG-URA3-hisG pdr3::hisG) strain was derivative of strain W303-1A. 8-mm paper discs containing each compound was placed on YPD agar medium containing YNS17 strain and 0.3 M CaCl$_2$. After 3 days of incubation at 28°C, the inhibitory activity of the Ca$^{2+}$-signaling transduction was determined by the diameter of the growth zone of the paper disc. FK506 (0.02 µg/disc) was used as a positive control. FK506 was kindly provided by Fujisawa Pharmaceutical Co., Ltd. (the present Astellas Pharma.
Inc., Tokyo Japan).

**Cell culture and cytotoxicity**

Rat basophilic leukemia RBL-2H3 cells (ATCC, Manasass, VA, USA) were maintained in DMEM supplemented with 10% heat-inactivated FBS (Sigma-Aldrich Co. LLC, St. Louis, MO, USA) and antibiotics [penicillin (50 units/ml)-streptomycin (50 µg/ml), Gibco, Thermo Fisher Scientific Inc., Waltham, MA, USA]. Cell viability was determined using 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay (Dojindo Lab., Kumamoto, Japan). RBL-2H3 cells were plated in triplicate at a concentration of 3×10^5 cells/well in a 96-well plate and incubated for overnight prior to treatment with various concentrations of 1 and 2 for 48 h. After 10 µl (5 mg/ml) of MTT was added to each well and it was incubated for 4 h at 37°C. Isopropanol (100 µl in 0.04 N HCl) was added to dissolve the MTT formazan and the optical density was measured at 560 nm with a microplate reader (Infinite F200 PRO, Tecan, Männedorf, Switzerland).

**Measurement of the degranulation (β-hexosaminidase (β-HEX) assay)**

RBL-2H3 cells were grown overnight in 96-well plates (3 x 10^5 cells/well) and sensitized with 0.5 µg/ml IgE (Yamasa Co., Tokyo, Japan) for 2 h. After washing with 200 µl Tyrode buffer (137 mM NaCl, 2.7 mM KCl, 1.0 mM MgCl₂, 1.8 mM CaCl₂, 5.6 mM D-glucose, 20
mM HEPES, 1 mg/ml BSA, pH7.3), cells were incubated in 98 μl of Tyrode buffer and 2 μl of each sample for 30 min, then stimulated with 10 μl of 1 μg/ml DNP-BSA (Merck Millipore Co., Billerica, MA, US) as an antigen for 10 min. RBL-2H3 cells were also stimulated with 10 μl of 0.2 μM tapsigargin (Sigma-Aldrich Co., St. Louis, MO, USA) for 20 min or 10 μl of 20 μM A23187 (Sigma-Aldrich Co.) for 30 min without the sensitization by IgE. The supernatant (30 μl) was then transferred to a 96-well plate and incubated with 70 μl substrate (1.3 mg/ml 4-nitrophenyl-N-acetyl-β-D-glucosaminide, Sigma-Aldrich Co.) in 0.1 M citrate buffer (pH 4.5) at 37℃ for 90 min. The reaction was stopped by adding 100 μl of stop solution (0.4 M glycine-NaOH). The absorbance was measured at 405 nm by a microplate reader. The inhibition (%) of β-hexosaminidase release by the sample was calculated using the following equation, and IC50 values were determined graphically. Inhibition (%) = [1-(T-N)/(C-N)] x 100%: Control (C) was Ig E+DNP-BSA (or thapsigargin or A23187) (+), test sample (-) and normal (N) was IgE+DNP-BSA (or thapsigargin or A23187) (-), test sample (-) and test (T) was Ig E+DNP-BSA (or thapsigargin or A23187) (+), test sample (+). Quercetin was used as a reference compound (IC50=5.7 μM)1.

**Calcineurin assay**

The calcineurin activity was measured using a commercial kit (AK-804, Biomol GmbH, Hamburg, Germany), in which free phosphate ion released from a substrate phosphopeptide (DLDVPIPGRFDRVRpSVAAE) was quantified by colorimetric analysis (650 nm) using the malachite green method. The calmodulin antagonist trifluoperazine was used as a positive control2.
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Figure S2. $^{13}$C NMR Spectrum of Compound 1 (125 MHz, CD$_3$OD)
Figure S3. DEPT Spectra of Compound 1 (125 MHz, CD₃OD)
Figure S4. COSY Spectrum of Compound 1 (600 MHz, CD$_3$OD)
Figure S5. HMOC Spectrum of Compound 1 (600 MHz, CD$_3$OD)
Figure S6. HMBC Spectrum of Compound 1 (600 MHz, CD$_3$OD)
Figure S7. $^1$H-$^1$H COSY (bold) and Key HMBC (arrows) correlations observed for 1.
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References

