The Journal of Antibiotics (2018) 71:626–632
https://doi.org/10.1038/s41429-018-0049-8

JARA Japan Antibiotics Research Association
The Society for Actinomycetes Japan

ARTICLE

Anti-*Rhizopus* activity of tanzawaic acids produced by the hot spring-derived fungus *Penicillium* sp. BF-0005

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Received: 8 December 2017 / Revised: 27 February 2018 / Accepted: 13 March 2018 / Published online: 1 May 2018
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Abstract

A silkworm infection assay with the pathogenic fungus *Rhizopus oryzae* was established. Microbial culture broths were screened for anti-*Rhizopus* antibiotics using this assay. A new compound, tanzawaic acid R was isolated along with known and structurally related tanzawaic acids and arohynapene A from the culture broth of the hot spring-derived fungus *Penicillium* sp. BF-0005. The structure of tanzawaic acid R was elucidated by various spectroscopic data including 1D and 2D nuclear magnetic resonance spectroscopy. Tanzawaic acids A, B, C, and R and arohynapene A exhibited antifungal activity against *R. oryzae*. Tanzawaic acids A and B dose-dependently exerted therapeutic effects in the silkworm infection assay with *R. oryzae*.

Introduction

Antibiotics against pathogenic microorganisms are generally screened using in vitro assay systems such as the paper disk and microdilution methods; however, in most cases, they show no therapeutic effects in in vivo assays using mammals because of pharmacokinetics, systemic absorption, and stability. In order to overcome these issues, an in vivo-mimic infection assay using the silkworm was established [1] and applied in the early stage of a screening process. We discovered nosokomycins and lysocins from microbial culture broths in the silkworm infection assay with methicillin-resistant *Staphylococcus aureus* [2, 3]. This in vivo-mimic screening system has been applied to other pathogenic microorganisms such as *Pseudomonas aeruginosa* and *Candida albicans* [4].

Zygomycosis, caused by zygomycetous fungi including *Rhizopus oryzae*, is an invasive opportunistic fungal infection that occurs in the setting of hematological malignancies, chemotherapy-induced neutropenia, and immunosuppressive therapies [5]; however, only amphotericin B (AMPB) has been clinically used as an anti-*Rhizopus* agent [5]. Therefore, new anti-*Rhizopus* antibiotics with different mechanisms of action are desired. Based on this background, we searched for new anti-*Rhizopus* antibiotics using an in vivo-mimic infection assay of silkworms. During this screening program, a new compound named tanzawaic acid R was isolated along with known and structurally related tanzawaic acids [6–10] and arohynapene A [11] from the culture broth of hot spring-derived *Penicillium* sp. BF-0005 (Fig. 1). In the present study, the silkworm infection assay as well as the fermentation, isolation, structural elucidation, and antifungal activity of tanzawaic acid R were described.

Results

Establishment of the silkworm infection assay with *R. oryzae*

Four different colony numbers of *R. oryzae* (6.75×10 to 6.75×10^4 CFU per larva g^{-1}, $n = 5$) were injected into silkworms and the silkworms were raised at 27 °C. As shown in Fig. 2, as the colony number increased, silkworms

Electronic supplementary material The online version of this article (https://doi.org/10.1038/s41429-018-0049-8) contains supplementary material, which is available to authorized users.

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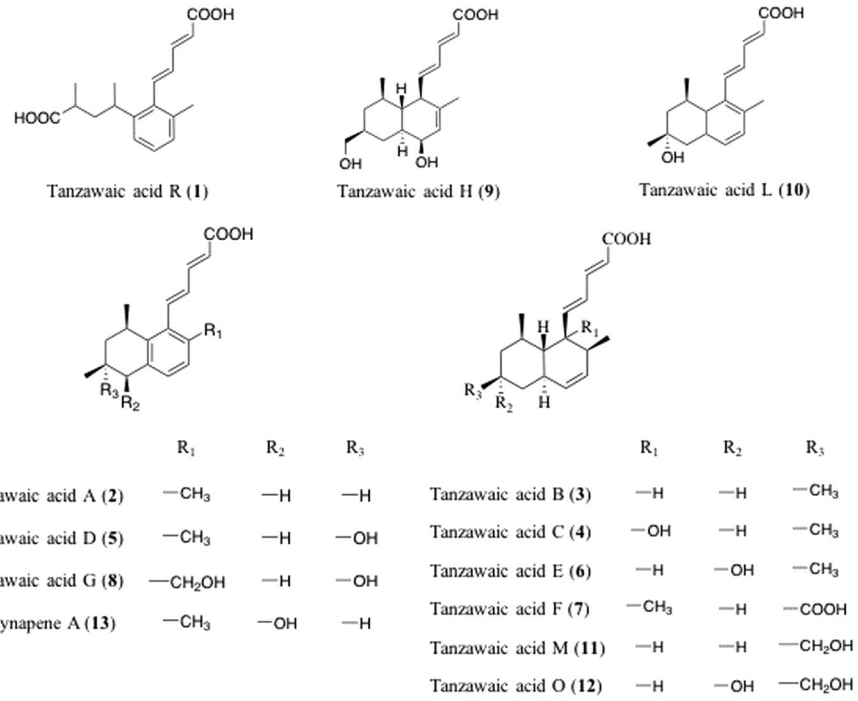
Fig. 1 Structures of **1** to **13**



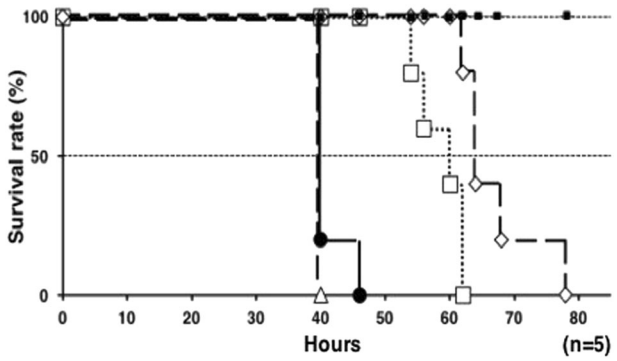


Fig. 2 Killing ability of *R. oryzae* against silkworms. A suspension of the *R. oryzae* NBRC4705 strain was diluted to the indicated cell number and administered into the silkworm hemolymph. Infected silkworms were incubated at 27 °C. The number of surviving silkworms was counted 60 h after the injection. Injected colony numbers of *R. oryzae*: ◊ 6.75×10, □ 6.75×10^2, ● 6.75×10^3, Δ 6.75×10^4 CFU per larva g^{-1}, and ■ without *R. oryzae*. Experiments were performed three times and reproducible data were observed

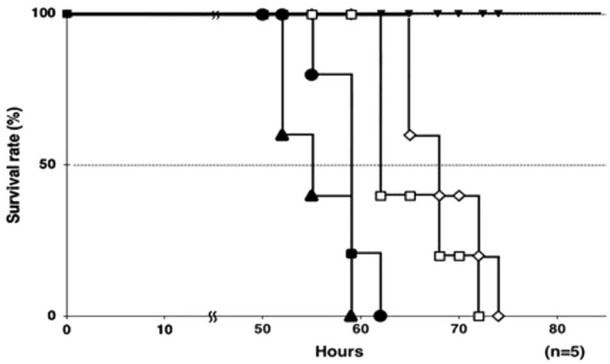


Fig. 3 Therapeutic effects of AMPB in the silkworm infection assay with *R. oryzae*. Doses of AMPB: Δ 0.675, ● 1.25, □ 2.5 ◊ 5.0 μg per larva g^{-1}, and ■ without *R. oryzae*. Experiments were performed three times and reproducible data were observed

started to die faster (40–64 h) and were all dead within a shorter time (40–78 h). Among the colony numbers employed in the present study, we used 6.75×10^2 CFU per larva g^{-1} for the screening assay. Under this condition, a clinical drug AMPB was effective, and survival of silkworms markedly increased (Fig. 3). These results proved therapeutic effects of AMPB in the silkworm infection assay.

Screening for new anti-*Rhizopus* antibiotics

We started screening for new anti-*Rhizopus* antibiotics; 8210 microbial culture broth samples were evaluated according to the broth microdilution method [12] in primary screening, and 213 reproduced samples were then evaluated in the silkworm infection assay in secondary screening. The therapeutic efficacies of culture samples of the three strains including the fungus BF-0005 were confirmed in the silkworm infection assay.

Fermentation of *Penicillium* sp. BF-0005

The fungus BF-0005, identified as *Penicillium* sp., was inoculated into a 500-mL Erlenmeyer flask containing 100 mL seed medium (2.0% glucose, 0.10% yeast extract, 0.050% $MgSO_4 \cdot 7H_2O$, 0.50% polypeptone, 0.10% KH_2PO_4, and 0.10% agar). The seed culture flask was shaken on a rotary shaker at 27 °C for 3 days. The seed culture (1.0 mL) was transferred to 26 × 1.0-L culture bottles containing 100 mL production medium (2.0% sucrose, 1.0% glucose, 0.50% Solulys, 0.50% meat extract, 0.10% KH_2PO_4, 0.30% $CaCO_3$, 0.10% agar, 1.0% trace metals, and 0.1% Yunohana, pH 3.0). Fermentation was performed under static conditions at 27 °C for 2 weeks.

Isolation

The culture broth (2.6 L) was treated with an equal volume of acetone. After the mixture was filtered and concentrated to remove acetone, the aqueous solution (1.0 L) was adjusted to pH 3.0 with 2 N HCl and extracted with EtOAc (2.0 L). The organic layer was dried over Na_2SO_4 and concentrated under reduced pressure to give a brown material (2.03 g). Crude materials were dissolved in a small amount of MeOH, applied to an octadecylsilyl column (60 g), and eluted stepwise with 0, 20, 40, 50, 60, 80, and 100% CH_3CN (40 mL × 6 each). The 40%-4 CH_3CN fraction containing **1**, **4**, **5**, and **9** was concentrated under reduced pressure to give a brown material (142 mg). Compounds **1**, **4**, **5**, and **9** were finally purified by preparative high-performance liquid chromatography (HPLC) under the following conditions: column, CAPCELL PAK C18 UG120 (i.d. 20 × 250 mm); mobile phase, 30% CH_3CN containing 0.05% H_3PO_4; detection, ultraviolet (UV) at 210 nm; flow rate, 8.0 mL min^{-1}. Under these conditions, **1**, **4**, **5**, and **9** were eluted as a peak with retention times of 42, 32, 29, and 36 min, respectively. After each fraction was concentrated to remove CH_3CN, aqueous solution was extracted with EtOAc. The organic layer was dried over Na_2SO_4 and concentrated under reduced pressure to give pure **1** (2.4 mg, white powder), **4** (4.5 mg, white powder), **5** (3.5 mg, white powder), and **9** (3.8 mg, white powder).

The 50%-5 CH_3CN fraction containing **6** and **12** was concentrated to give a brown material (41.8 mg). Compounds **6** and **12** were also purified by preparative HPLC: column, CAPCELL PAK C18 UG120 (i.d. 20 × 250 mm); mobile phase, 40% CH_3CN containing 0.05% H_3PO_4; detection, UV at 210 nm; flow rate, 8.0 mL min^{-1}. Under these conditions, **6** and **12** were eluted as a peak with retention times of 24 and 29 min, respectively. Fractions were treated in a similar manner to give pure **6** (4.3 mg, yellow oil) and **12** (4.0 mg, white powder).

The 60%-1 CH_3CN fraction containing **7**, **8**, and **11** was concentrated to give a brown material (16.3 mg). Compounds **7**, **8**, and **11** were purified by preparative HPLC: column, CAPCELL PAK C18 UG120 (i.d. 20 × 250 mm); mobile phase, 45% CH_3CN containing 0.05% H_3PO_4; detection, UV at 210 nm; flow rate, 8.0 mL min^{-1}. Under these conditions, **7**, **8**, and **11** were eluted as a peak with retention times of 29, 31, and 36 min, respectively. Pure **7** (2.5 mg, yellow oil), **8** (5.8 mg, white powder), and **11** (6.2 mg, yellow oil) were obtained from these fractions.

The 60%-4 CH_3CN fraction containing **10** and **13** was concentrated to give a brown material (38.5 mg). Compounds **10** and **13** were purified by preparative HPLC under the same conditions as those for **7**, **8**, and **11**. Compounds **10** and **13** were eluted as a peak with retention times of 38 and 40 min, respectively, yielding pure **10** (3.5 mg, colorless oil) and **13** (3.0 mg, yellow powder).

The 80%-5 CH_3CN fraction containing **2** was concentrated under reduced pressure to give a white material (35.3 mg). Compound **2** was finally purified by preparative HPLC: column, CAPCELL PAK C18 UG120 (i.d. 20 × 250 mm); mobile phase, 40 min gradient from 55% CH_3CN-0.05% H_3PO_4 to 85% CH_3CN-0.05% H_3PO_4; detection, UV at 210 nm; flow rate, 8.0 mL min^{-1}. Under these conditions, compound **2** was eluted as a peak with a retention time of 29 min, yielding pure **2** (13.2 mg) as a colorless oil.

The 100%-1 CH_3CN fraction containing **3** was concentrated to give a colorless oil (19.8 mg).

Structural elucidation

The physicochemical properties of **1** are summarized in Table 1. Compound **1** was obtained as a white powder. In UV spectra, compound **1** showed absorption maxima at 278, 218, 233, and 240 nm. Infrared (IR) absorption at 3264 cm^{-1} and 1710–1732 cm^{-1} suggested the presence of

Table 1 Physicochemical properties of **1**

Appearance	White powder
Molecular formula	$C_{18}H_{22}O_4$
Molucular weight	302
HR-ESI-MS (*m/z*)	
Calcd	325.14158 $[M + Na]^+$
Found	325.14301 $[M + Na]^+$
UV (MeOH) λmax (nm) (log ε)	218 (3.35), 233 (3.31), 240 (3.31), 278 (3.45)
IR (KBr) vmax (cm^{-1})	3290, 2950, 1732, 1710, 1600, 1420, 1125
$[\alpha]_D^{23}$ ($c = 0.1$, MeOH)	+34.3

HR-ESI-MS high-resolution electrospray ionization-mass spectrometry, *UV* ultraviolet, *IR* infrared

hydroxyl and carbonyl groups in the structures. The molecular formula of **1** was assigned to $C_{18}H_{22}O_4$ on the basis of high-resolution electrospray ionization-mass spectrometry (ESI-MS) measurements ([*m/z* 325.1430 $(M + Na)^+$ *m/z* $\Delta + 1.43$ mmu]).

The ^{13}C nuclear magnetic resonance (NMR) spectrum of **1** showed 18 carbon signals, which were classified into three methyl, one sp^3 methylene, two sp^3 methine, seven sp^2 methine, three sp^2 quaternary, and two carbonyl carbons by distortionless enhancement by polarization spectra. The ^1H NMR spectrum of **1** displayed 20 proton signals, 6 of which were assigned to three methyl protons (δ 1.07, 1.20, and 2.28) and three aromatic methine protons (δ 7.04, 7.12, and 7.13). The connectivity of all proton and carbon atoms was established by heteronuclear single quantum coherence experiments (Table 2). As shown by the bold line in Fig. 4, three partial structures, **I**, **II**, and **III**, were elucidated by ^1H-^1H correlation spectroscopy spectra. Furthermore, the ^{13}C-^1H long-range couplings of ^2J and ^3J in the heteronuclear multiple bond correlation spectra (Fig. 4) proved the presence of the following linkages: (1) The cross-peaks from sp^2 methine proton H-8 (δ 7.04) to sp^2 quaternary carbon C-7 (δ 136.8), from sp^2 methine proton H-9 (δ 7.13) to C-7 and sp^2 quaternary carbon C-11 (δ 146.2), from sp^2 methine proton H-10 (δ 7.12) to sp^2 quaternary carbon C-6 (δ 136.9) and C-11, and from methyl protons 16-CH_3 (δ 2.28) to C-6, C-7, and sp^2 methine carbon C-8 (δ 128.8) suggested the presence of a trisubstituted benzene ring with a methyl group at the C7 position containing the partial structure III. (2) The cross-peaks from sp^2 methine proton H-2 (δ 5.98) and sp^2 methine proton H-3 (δ 7.35) to carboxyl carbon C-1 (δ 171.8) suggested the presence of a penta-2,4-dienoic acid chain containing the partial structure **II**. Furthermore, the cross-peaks from sp^2 methine proton H-4 (δ 6.35) to C-6 and from sp^2 methine proton H-5 (δ 7.03) to C-6, C-7, and C-11 indicated a connection between C-5 and C-6, revealing that penta-2,4-dienoic acid was attached to the C-6 position. (3) The cross-peaks from sp^2 methylene protons H-13 (δ 1.97 and 1.53), sp^3 methine proton H-14 (δ 2.30), and the methyl protons 15-CH_3 (δ 1.07) to carboxyl carbon C-18 (δ 181.6) suggested the presence of 2-methyl-pentanoic acid containing the partial structure **I**. Furthermore, the cross-peaks from sp^3 methine H-12 (δ 3.13) to C-6, sp^2 methine carbon C-10 (δ 124.3), and C-11, and from methyl protons 17-CH_3 (δ 1.20) to C-11 indicated that this part is connected to the benzene ring at the C-11 position. Taken together, the structure of **1** was elucidated as shown in Fig. 1 since this structure fulfilled the molecular formula $C_{18}H_{22}O_4$ and eight degrees of unsaturation. The geometry of two double bonds was determined by an analysis of coupling constants. The large coupling constants of H-2/H-3 and H-4/H-5 (Table 2) indicated that the geometries of the double bonds at the C-2 and C-4 positions were the *E* configuration.

Table 2 ^1H and ^{13}C NMR chemical shifts of **1** in MeOH-d_4

Position	δC	δH	
1	171.8		
2	125.9	5.98 (d, 15.3)	1H
3	143.9	7.35 (dd, 15.3, 11.0)	1H
4	134.4	6.35 (dd, 15.3, 11.0)	1H
5	138.5	7.03 (d, 15.3)	1H
6	136.9		
7	136.8		
8	128.8	7.04 (d, 7.8)	1H
9	128.7	7.13 (dd, 7.8, 8.0)	1H
10	124.3	7.12 (d, 8.0)	1H
11	146.2		
12	34.0	3.13 (m)	1H
13	43.4	1.53 (dd, 13.8, 6.3), 1.97 (ddd, 13.8, 7.8, 2.8)	2H
14	39.2	2.30 (m)	1H
15	18.0	1.07 (d, 6.7)	3H
16	22.2	2.28 (s)	3H
17	21.4	1.20 (d, 6.6)	3H
18	181.6		

NMR nuclear magnetic resonance

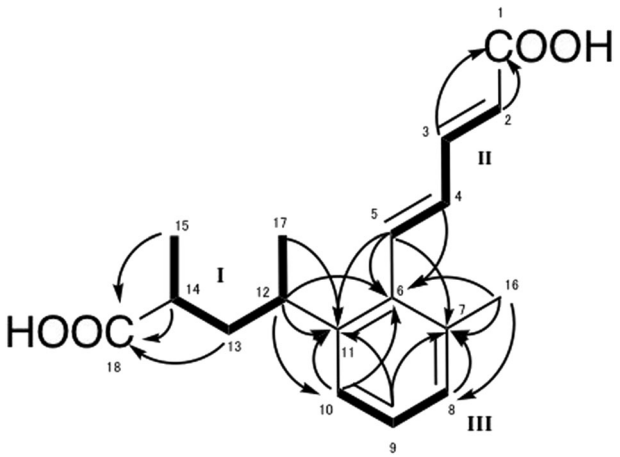


Fig. 4 Key correlations in correlation spectroscopy (COSY) and heteronuclear multiple bond correlation (HMBC) spectra of **1**

The structures of compounds **2** to **13** were similarly elucidated by NMR experiments. They were identified as tanzawaic acids by comparisons with previous NMR data (Tables S1–S20).

In vitro antifungal activity

Antifungal activities of **1** to **13** and AMPB against four pathogenic fungi, *R. oryzae*, *C. albicans*, *Cryptococcus*

Table 3 Antifungal activities of **1** to **13** against *R. oryzae*

Test compound	*R. oryzae* NBRC4705	Test compound	*R. oryzae* NBRC4705
1	32	**8**	>64
2	1.0	**9**	>64
3	0.5	**10**	>64
4	16	**11**	>64
5	>64	**12**	>64
6	>64	**13**	8.0
7	>64	AMPB	1.0

AMPB amphotericin B

neoformans, and *Aspergillus fumigatus*, were evaluated according to the broth microdilution method. Compounds **1**, **2**, **3**, **4**, and **13** exhibited antifungal activities against *R. oryzae* with minimal inhibitory concentration (MIC) values of 32, 1.0, 0.5, 16, and 8.0 μg mL^{-1}, respectively (Table 3). Meanwhile, the antifungal activities of compounds **1** to **13** against *C. albicans*, *C. neoformans*, and *A. fumigatus* were not observed (the MIC values: >64 μg mL^{-1}), and AMPB had broad antifungal activities against *R. oryzae*, *C. albicans*, *C. neoformans*, and *A. fumigatus* with MIC values of 1.0, 0.5, 1.0, and 4.0 μg mL^{-1}, respectively. These results indicated that compounds **1**, **2**, **3**, **4**, and **13** had selective activities towards *R. oryzae*. Notably, compounds **2** and **3** had almost the same potency as AMPB.

Therapeutic efficacy in the silkworm infection assay with *R. oryzae*

Compounds **2** and **3** were evaluated in the silkworm infection assay with *R. oryzae* ($n = 5$). After being infected with *R. oryzae*, all silkworms died within 60 h. Under this condition, the injection of **2** and **3** dose-dependently increased the survival of silkworms (Fig. 5a, b). At the maximal dose of **2** and **3**, silkworms survived longer than 12 h. Furthermore, 50% effective dose (ED_{50}) values of these compounds are summarized in Table 4. This result indicated that both **2** and **3** exerted therapeutic effects with the ED_{50} values of 7.0 μg per larva g^{-1}.

Discussion

An in vivo-mimic assay of silkworms with *R. oryzae* was established in the present study. We previously reported that a colony number of 1.0×10^6 CFU per larva g^{-1} was needed for *C. albicans*-infected silkworms to die [4]. In contrast, *R. oryzae* killed silkworms at a lower colony number of 6.5×10^2 CFU per larva g^{-1}. The killing ability of *R. oryzae* against silkworms was shown to be higher than that of

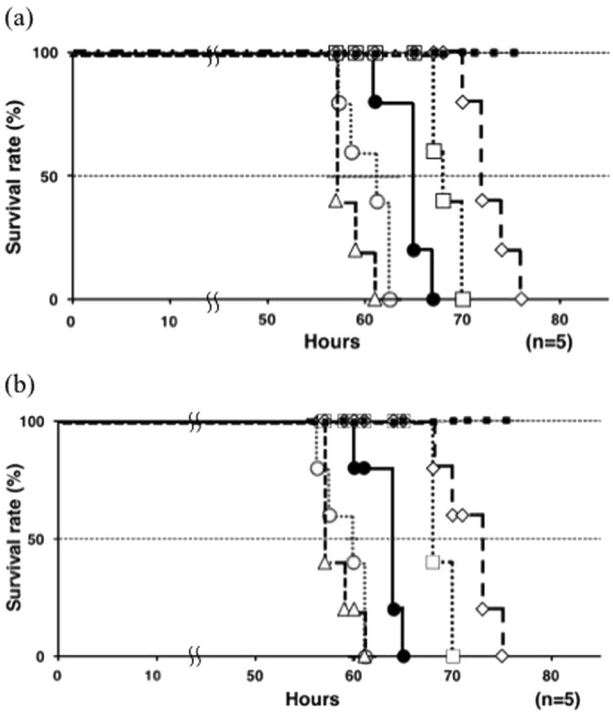


Fig. 5 Therapeutic effects of **2** and **3** in the silkworm infection assay with *R. oryzae*. Doses of **2** (**a**) and **3** (**b**): Δ 0, ○ 6.25, ● 12.5, □ 25, ◊ 50 μg per larva g^{-1}, and ■ without *R. oryzae*. Experiments were performed three times and reproducible data were obtained

Table 4 ED_{50} values of **2**, **3**, and AMPB in the silkworm infection assay with *R. oryza*

Test compound	ED_{50} (μg g^{-1} larva)
2	7.0
3	7.0
AMPB	0.65

ED_{50} 50% effective dose, *AMPB* amphotericin B

C. albicans. This may reflect different degrees of pathogenicity and virulence between *R. oryzae* and *C. albicans*. As expected, AMPB, a clinically important antifungal drug, dose-dependently increased the survival of silkworms in both assay models of *R. oryzae* and *C. albicans*. The therapeutic efficacy of AMPB proved the usefulness of this silkworm assay for the evaluation of antifungal agents.

By applying this silkworm infection assay to the screening of new anti-*Rhizopus* antibiotics, microbial samples could be efficiently prioritized, as shown by the hit rates. Further isolation studies on one fungal strain, BF-0005 led to the discovery of 13 compounds related to tanzawaic acids including one new compound. Hot spring-derived mineral components called "Yunohana" were required for their production; when the fungus was fermented in the absence of the components, they were not observed.

Furthermore, the broth microdilution assay indicated that only five compounds showed moderate antifungal activities, whereas others had no activity. Potency was in the order of **3** > **2** > **13** > **4** > **1** (Table 3). Notably, other inactive compounds possessed one or two hydroxyl groups or one carboxyl group in the tetrahydro/octahydro-naphthalene ring. Regarding antifungal activity, it may have been unfavorable for such a hydrophilic functional group to be positioned adjacent to the bicyclic ring.

Interestingly, in vitro potency of compounds **2** and **3** was estimated to be the same as AMPB, whereas their in vivo therapeutic effects were approximately 10-fold weaker than AMPB. Pharmacokinetics of these compounds in the silkworms is predicted to affect in vivo activities [3], and may cause the difference of activities between the two assays.

Tanzawaic acids were first isolated in 1999 from the fungus *Penicillium citrinum* [6]. *Penicillium* genus fungi were subsequently reported to produce some types of compounds belonging to this family; 19 tanzawaic acids have been identified to date [6–11]. While this family typically exhibits several biological activities such as antibacterial and antioxidant activities and cell cytotoxicity, antifungal activity against *R. oryzae* is not described in previous studies. To the best of our knowledge, this is the first study on the antifungal activity of this family against *R. oryzae*. Moreover, another interesting result is the more selective activity of tanzawaic acids towards *R. oryzae* among the four pathogenic fungi tested. They may act on target molecules that are characteristic of zygomycetous fungi, but not of *Candida*, *Aspergillus*, and *Cryptococcus*. Therefore, the identification of target molecules is expected to provide new targets for the development of drugs for zygomycosis.

In conclusion, we established a silkworm infection assay with *R. oryzae*, applied this assay to a screening program of new anti-*Rhizopus* antibiotics, and discovered tanzawaic acids A and B with moderate therapeutic efficacies in the silkworm model. They clearly have the ability to increase the survival of silkworms from a lethal infection with *R. oryzae* in a dose-dependent manner, similar to AMPB. These compounds have potential in drug development for zygomycosis.

Materials and methods

Materials

Glucose, sucrose, polypeptone, KH_2PO_4, $CaCO_3$, and $Mg_3(PO_4)_2$•$8H_2O$ were purchased from Wako Pure Chemical Industries (Osaka, Japan). $MgSO_4$•$7H_2O$ was purchased from Kanto Chemical (Tokyo, Japan). Yeast extract, potato dextrose agar (PDA), and potato dextrose broth (PDB) were purchased from Becton Dickinson (Sparks, MD, USA). Solulys was purchased from ORIENTAL YEAST (Tokyo, Japan). Meat extract was purchased from Kyokuto Pharmaceutical Industrial (Tokyo, Japan). Yunohana was purchased from Hakone Onsen Kyokyu (Kanagawa, Japan). RPMI 1640 medium was purchased from Life Technologies (Carlsbad, CA, USA). CAPCELL PAK C18 UG120 was purchased from Shiseido (Tokyo, Japan) for analyses using HPLC. AMPB was purchased from Sigma Aldrich (St Paul, MN, USA). Fertilized silkworm eggs, *Bombyx mori* (Hu•yo × Tukuba•Ne), were purchased from Ehime Sanshu (Ehime, Japan). Silk Mate 2S, an artificial diet containing antibiotics, was purchased from Nosan Corporation (Kanagawa, Japan).

The following fungal strains were used in this study: *R. oryzae* NBRC4705, *C. albicans* ATCC90029, *C. neoformans* ATCC90013, and *A. fumigatus* NBRC33022.

General experimental procedure

ESI-MS spectrometry was conducted on a JMS-T100LP spectrometer (JEOL, Tokyo, Japan). UV and IR spectra were measured with a U-2800 spectrophotometer (Hitachi, Tokyo, Japan) and FT/IR-460 plus spectrometer (JASCO, Tokyo, Japan), respectively. The ^{13}C (100 MHz) and ^{1}H (400 MHz) spectra of compound **1** were taken on the XL-400 NMR system (Agilent, Santa Clara, CA, USA) in MeOH-d_4, and the solvent peak was used as an internal standard at 3.31 ppm for ^{1}H NMR and 49.0 ppm for ^{13}C NMR. The ^{1}H (400 MHz) spectra of compounds **2** to **13** were taken in MeOH-d_4, $CHCl_3$-d, and dimethyl sulfoxide-d_6 (DMSO-d_6), and the solvent peak was used as an internal standard at 7.26 ppm for $CHCl_3$-d and 2.48 ppm for DMSO-d_6.

Fungal strain and identification

The fungus BF-0005 was isolated from a soil sample collected in Hakone Owakudani (Kanagawa, Japan). The genus was identified based on a genetic analysis of a rDNA internal transcribed spacer according to the established method [13].

Assay for antifungal activity using the microdilution method

R. oryzae and *A. fumigatus* were subcultured on PDA at 27 °C. Colonies that sporulated were collected with sterile 0.85% saline (2.0 mL), a homogeneous suspension containing spores and conidia was left to stand for 5 min in order to settle heavy particles, and the upper homogeneous suspension was then transferred to a sterile tube and

adjusted at McFarland 0.5. The inocula for homogeneous suspensions were diluted to 1/50 with RPMI 1640 medium. The MIC values of samples against *R. oryzae* and *A. fumigatus* were assessed using the broth microdilution assay according to the standard guidelines described in the Clinical and Laboratory Standards Institute (CLSI) documents M38-A2 method [13].

C. albicans and *C. neoformans* were subcultured on PDA plates at 27 °C. Five colonies of ~1 mm in diameter on PDA after a 48-h incubation at 27 °C were suspended in sterile 0.85% saline (5.0 mL), and the turbidity of the suspension was adjusted at McFarland 0.5. The suspension was diluted to 1/2000 with RPMI 1640 medium. The MIC values of samples against *C. albicans* and *C. neoformans* were assessed in the broth microdilution assay according to the standard guidelines described in the CLSI documents M27-A3 method [14].

Silkworm infection assay with *R. oryzae*

An *R. oryzae* suspension was adjusted at McFarland 0.5 in a similar manner. The inocula for homogeneous suspensions were diluted to 1/100 in sterile 0.85% saline (2.7×10^4 CFU mL^{-1}). Hatched silkworm larvae were raised by feeding Silk Mate 2S in an incubator at 27 °C until the fourth molting stage. On the first day of fifth-instar larvae, silkworms were fed Silk Mate 2S. On the second day, *R. oryzae* (1.35×10^3 CFU in 50 µL of sterile 0.85% saline) was injected into the hemolymph through the dorsal surface of the silkworm using a disposable 1-mL syringe with a 27-G needle (TERUMO, Tokyo, Japan). Samples solubilized in 50 µL of 50% DMSO were injected into the hemolymph within 1 h of the infection with the fungus. All *R. oryzae*-infected silkworms died within 60 h when no sample was administered. After the sample injection, the silkworms that survived were counted at the indicated time until 80 h to calculate the survival rate of each sample. Furthermore, the survival rate at the indicated dose of each sample was also calculated when all *R. oryzae*-infected silkworms without the injection of samples died. The ED_{50} values were calculated from each graph as shown in Fig. S21 according to the previous method [15, 16].

Acknowledgements We thank N Sato and Dr. K Nagai, School of Pharmacy, Kitasato University, for measurements of NMR and mass spectra, respectively. This work was supported by JSPS KAKENHI Grant Numbers 16H05095 (to RU) and 21310146 (to HT), and the Institute for Fermentation, Osaka (to RU).

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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