Traminines A and B, produced by Fusarium concentricum, inhibit oxidative phosphorylation in Saccharomyces cerevisiae mitochondria

Katsuyuki Sakai1, Yufu Unten2, Aoi Kimishima1,3, Kenichi Nonaka1,3, Takumi Chinen4, Kazunari Sakai1,3, Takeo Usui5,4, Kazuhiro Shiomi1,3, Masato Iwatsuki2, Hideto Miyoshi4, Satoshi Ōmura3

1Graduate School of Infection Control Sciences, Kitasato University, 5-9-1 Shirokane, Minato-ku, Tokyo 108-8641, Japan
2Division of Applied Life Sciences, Graduate School of Agriculture, Kyoto University, Kitashirakawa Oiwake-cho, Sakyo-ku, Kyoto 606-8502, Japan
3Ōmura Satoshi Memorial Institute, Kitasato University, 5-9-1, Shirokane Minato-ku, Tokyo 108-8641, Japan
4Graduate School of Life and Environmental Sciences, University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki 305-8572, Japan

Correspondence should be addressed to: Yukihiro Asami. E-mail: yasami@lisci.kitasato-u.ac.jp

Abstract: Two new tetramic acid derivatives, traminines A (1) and B (2), were isolated from a culture broth of Fusarium concentricum FKI-7550 by bioassay-guided fractionation using multidrug-sensitive Saccharomyces cerevisiae Δ12geneΔHOSR-iERG6. The chemical structures of 1 and 2 were elucidated by NMR studies. Compounds 1 and 2 inhibited the growth of the multidrug-sensitive yeast strain on nonfermentable medium containing glycerol, but not on fermentable medium containing glucose. These results strongly suggest that they target mitochondrial machineries presiding over ATP production via oxidative phosphorylation. Throughout the assay monitoring overall ADP-uptake/ATP-release in yeast mitochondria, 1 and 2 were shown to inhibit one or more enzymes involving oxidative phosphorylation. Based on biochemical characterization, we found that the interference with oxidative phosphorylation by 1 is attributable to the dual inhibition of complex III and F1F0-ATPase, whereas that by 2 is solely due to the inhibition of complex III.

Keywords: Saccharomyces cerevisiae, Multidrug-sensitive, Mitochondria

Introduction

Microorganisms produce secondary metabolites that have tremendous diversity in chemical structures, biological activities, and target proteins. These natural products potentially have numerous applications in the research and development of pharmaceuticals and agrochemicals. Leucomycin, staurosporine, and avermectin are well-known examples of such products, which have been exploited as drugs in clinical practice (Newman & Cragg, 2020; Ōmura et al., 2018).

The mitochondrial machineries presiding over ATP synthesis via oxidative phosphorylation are promising targets of biologically active compounds derived from microbial metabolites. A variety of naturally occurring chemicals have been reported to inhibit mitochondrial respiratory enzymes and F1F0-ATPase, some of which are being investigated as excellent drug candidates (Bald et al., 2017; Kita et al., 2007; Weinberg & Chandel, 2015). In this study, we conducted a comprehensive screening for natural products targeting the mitochondrial machineries using a yeast-screening system.

This screening system uses the budding yeast Saccharomyces cerevisiae, which is useful for identifying and evaluating biologically active compounds. However, the high drug resistance of the yeast is often an obstacle in discovering new compounds. One factor in the mechanism of drug resistance in S. cerevisiae is the ATP-binding cassette (ABC) transporter family, which effluxes exogenously added compounds from the cells. Therefore, the use of S. cerevisiae lacking the genes of ABC transporters was expected to be useful for the discovery of biologically active compounds that could not be identified by existing methods. In this work, the S. cerevisiae Δ12geneΔHOSR-iERG6 strain (Chinen et al., 2014), a multidrug-sensitive strain in which 12 genes related to ABC transporters have been disrupted, was introduced into the screening system. We selected microbial culture broths, in which the growth of the S. cerevisiae Δ12geneΔHOSR-iERG6 was inhibited in glycerol-containing medium (YPD agar medium; 1% yeast extract, 2% peptone, 3% glucose, and 1.5% agar) but not in glucose-containing medium (YPG agar medium; 1% yeast extract, 2% peptone, 3% glycerol, and 1.5% agar). This approach using two different carbon sources has been used to isolate compounds targeting mitochondrial oxidative phosphorylation (Sakai et al., 2019; Suga et al., 2015; Watanabe et al., 2017). Based on the above strategies, we revealed that Fusarium concentricum FKI-7550 produced two new tetramic acid derivatives; namely, traminines A (1) and B (2). Here, we report the fermentation, isolation, structural determination, and biological profiles of these new compounds.

Materials and Methods

General Experiments

Reverse- and normal-phase column chromatography was conducted on YMC-gel ODS-A (150 μm; YMC Co., Ltd., Kyoto, Japan) and Silica gel 60 (0.063–0.200 mm; Merck KGaA, Darmstadt, Germany), respectively. A Chromatorex C8 SPS100-SHE column was purchased from Fuji Silysia Chemical Co. (Aichi, Japan). High- and low-resolution mass data were measured on a JEOL JMS-T100LP (JEOL, Tokyo, Japan). NMR spectra were measured on a Bruker Avance III HD600 (Bruker Corp, Billerica, MA, USA) with
Fusarium concentricum
by measuring OD600 on a Corona Grating Microplate Reader SH-
0.1% KH2PO4, and 0.1% agar, pH 6.0] and incubated on a rotary
Polypeptone (FUJIFILM Wako Pure Chemical Co., Osaka, Japan),
centricum (NITE-2944 [Sakai et al., 2019]). One loopful of strain
KCl, 0.2% NaNO3, and 1.5% agar, pH 6.0) was inoculated into a
Tokyo, Japan).
was measured on a JASCOP-2200 polarimeter (JASCOCorporation,
spectrophotometer (Hitachi Ltd., Tokyo, Japan). Optical rotation
DMSO-
DMSO-
CA, USA) with 1H NMR at 400 MHz and 13C NMR at 100 MHz in
Varian XL-400 spectrometer (Agilent Technologies, Santa Clara,
Fusarium concentricum from soil around the root of Cerasus × yedoensis collected in Tokushima, Japan. We have already reported that the fusaramin producing strain FKI-7550 was assigned to the genus and was designated as Fusarium concentricum (NITE-2944 [Sakai et al., 2019]). One loopful of strain Fusarium concentricum FKI-7550 grown on an LcA slant (0.1% glyc-
erol, 0.08% KH2PO4, 0.02% K2HPO4, 0.02% MgSO4·7H2O, 0.02% KCl, 0.2% NaNO3, and 1.5% agar, pH 6.0) was inoculated into a
500 ml-Erlenmeyer flask containing 100 ml of a pre-seed culture
medium [2% glucose, 0.2% yeast extract, 0.05% MgSO4·7H2O, 0.5%
Polypeptone (FUJIFILM Wako Pure Chemical Co., Osaka, Japan),
0.1% KH2PO4, and 0.1% agar, pH 6.0] and incubated on a rotary
shaker at 27°C for 4 days. Fifty-hundred ml of seed culture was
prepared by the pre-seed culture medium. Twenty-five ml of the
seed culture was inoculated into each of 200 Ulpack 47 culture
bags (Hokken Co., Ltd., Tochigi, Japan) containing a production
medium (500 g of water-sodden rice); Static fermentation was con-
tinued at 27°C for 12 days.

Evaluation of MIC Values of 1 and 2
We evaluated the minimum inhibitory concentration (MIC) of
each compound against yeasts using a broth microdilution
method (Sakai et al., 2019). The test compounds were dissoluted in
MeOH. The yeasts (1.0 × 10^6 cells/100 μL/well in a 96-well plate)
were cultured in either YPD or YPG liquid medium with 0.00001%
TWEEN 20 (Sigma-Aldrich, Co., St. Louis, MO, USA) in the pres-
ence of various concentrations of 1 and 2 for 2 and 3 days, respec-
tively. Minimum inhibitory concentration values were evaluated by
measuring OD600 on a Corona Grating Microplate Reader SH-
9000 (Corona Electric, Ibaraki, Japan).

Yeast Culture and Isolation of Mitochondria
S. cerevisiae W303-1B (MATa ade2-1 leu2-3,112 his3-2,215 trp1-
1 ura3-1 can1-100) cells were grown in semisynthetic lactate
medium, and mitochondria were isolated by digesting the cell wall
with Zymolyase-20T following by homogenization and differen-
tial centrifugation, as described previously (Glick & Pon, 1995; Un-
ten et al., 2019). The final mitochondrial pellet was resuspended
in buffer containing 0.60 M mannitol, 10 mM Tris/HCl (pH 7.4),
0.1 mM EDTA, 0.1% BSA, and protease inhibitor cocktail (Sigma-
Aldrich). Protein concentrations were determined using the BCA
Protein Assay Kit (Thermo Fisher Scientific, Maltham, MA, USA)
with BSA as the standard.

Measurement of ADP-Uptake/ATP-Release in
Yeast Mitochondria
The measurement of ADP-uptake/ATP-release in isolated yeast
mitochondria was conducted according to the procedures as de-
scribed previously (Unten et al., 2019). Freshly prepared mitochon-
dria (50 μg of proteins/ml) were suspended in 2.5 ml of reaction
buffer (0.60 M mannitol, 0.10 mM EGTA, 2.0 mM MgCl2, 10 mM
KPi, 5.0 mM α-ketoglutarate, and 10 mM Tris-HCl (pH 7.4) at 30°C
in the presence of an ATP-detecting system (2.5 mM glucose, hex-
okinase [1.7 Enzyme Units (EU)], glucose-6-phosphate dehydroge-
nase (0.85 EU), 0.20 mM NADP+, and 10 μM ApoA [a specific inhib-
itor of mitochondrial adenylate kinase]). Externally added ADP
(100 μM) started the exchange reaction with ATP synthesized in
the mitochondrial matrix. The formation of NADPH, which is pro-
portional to ATP efflux, was monitored spectrophotometrically for
10 min at 340 nm (ε = 6.2 mM−1 cm−1) with a Shimadzu UV-3000
(Shimadzu Co., Kyoto, Japan).

Measurement of Respiratory Enzyme and
FoF1-ATPase Activities in Yeast Mitochondria
For the assays of each respiratory complex, yeast mitochondria were
permeabilized by repeated freeze thawing in 50 mM KPi buffer
(pH 7.4) to improve the accessibility of substrates, such as
NADH, ATP, and cytochrome c (cyst. c) (Kelso et al., 2001; Un-
ten et al., 2019). NADH-Q oxidoreductase activity (NDH-2) was fol-
lowed by the oxidation of NADH (340 nm; ε = 6.2 mM−1 cm−1) in
buffer (2.5 ml) containing 50 mM KPi (pH 7.4), 0.4 μM antimycin A
4.0 mM KCN, and 100 μM ubiquinone-1 (Q1) at 30°C. The protein
concentration was set to 12 μg/ml, and the reaction was initiated
by the addition of NADH (final 50 μM).

Succinate-cyt. c oxidoreductase activity (complexes II–III) was fol-
lowed by the reduction of cyst. c (550–540 nm; ε = 21 mM−1 cm−1)
in buffer (2.5 ml) containing 50 mM KPi (pH 7.4), 4.0 mM KCN, and
50 μM cyst. c (from horse heart, Sigma-Aldrich) at 30°C. The protein
concentration was set to 12 μg/ml, and the reaction was initiated
by the addition of sodium succinate (final 5.0 mM). NADH-cyt. c
oxidoreductase activity (NDH-2-complex III) was followed by the
same conditions using 50 μM NADH as an electron donor.

Cytochrome c oxidase activity (complex IV) was followed by
the oxidation of reduced cyst. c (550–540 nm; ε = 21 mM−1 cm−1)
in 50 mM KPi buffer containing 0.5 μM antimycin A (pH 7.4, 2.5 ml)
at 30°C. The protein concentration was set to 12 μg/ml, and the reac-
tion was initiated by the addition of dithionite-reduced cyst. c
(final 30 μM).

Hydrolysis of ATP FoF1-ATPase [complex V] was measured in
2.5 ml of reaction buffer containing 50 mM Tris/HCl (pH 8.0),
0.50 μM antimycin A, and 6.0 mM MgCl2 at 30°C in the pres-
ence of ATP-generating system (2.0 mM 2-phosphoenolpyruvate,
50 μl of a pyruvate kinase/lactate dehydrogenase mixture [Sigma-
Aldrich], and 100 μM NADH). The protein concentration was set
to 12 μg/ml, and the reaction was initiated by the addition of ATP
(final 4.0 mM). The oxidation of NADH, which is proportional to
ATP hydrolysis, was monitored spectrophotometrically at 340 nm
(ε = 6.2 mM−1 cm−3).

Measurement of Complex III Activity in Bovine
Heart Submitochondrial Particles
Bovine submitochondrial particles (SMPs) were prepared by the
method of Matsumo-Yagis and Hatefi (Matsumo-Yagi & Hatefi,
1985), and complex III activity was followed by the reduction of cyst. c
with a Shimadzu UV-3000 (550–540 nm, ε = 21 mM−1 cm−1) us-
ing n-decylubiquinol (DBH2) as an electron donor (Yabunaka et al.,
2002). To improve the accessibility of cyst. c, SMPs (7.5 mg/ml) were
reated with sodium deoxycholate (0.9% (wt/vol), 1 h, on ice) be-
fore dilution with reaction medium. Then, the SMPs (10 μg of
proteins/ml) were incubated with an inhibitor for 4 min in a re-
action medium (2.5 ml) containing 0.25 M sucrose, 1.0 mM MgCl2,
4 mM KCN, 60 μM cyst. c (from horse heart), and 50 mM KPi (pH
7.4). The reaction was initiated by the addition of 50 μM DBH2.
The redox statuses of cytochromes b (containing hemes b1 and b1) and c (containing heme c1) were monitored spectrophotometrically at 563–575 and 539–553 nm, respectively (Link et al., 1993; Matsuno-Yagi & Hatefi, 1996). The absorbance change was recorded with a Shimadzu UV-3000 spectrophotometer in a dual wavelength mode. Bovine SMPs (1.2 mg/ml) were suspended in 20 mM reaction buffer (250 mM sucrose, 50 mM KPi, 1.0 mM MgCl2, 4.0 mM KCN, and 0.2 μM SF6847, pH 7.4) at 30°C and the reaction was initiated by the addition of 5 mM sodium succinate after the inhibition of complex III activity. Complete reduction of the hemes was achieved by the addition of excess sodium dithionite in the cuvette.

**Results and Discussion**

**Extraction and Isolation of 1 and 2**

The stationary culture (100 kg) was extracted with acetone (120 l) and the extract was filtrated. The filtrate was concentrated in vacuo to remove acetone. The remaining aqueous solution (35 l) was applied to a silica gel column and eluted stepwise with a mixture of CHCl3 - MeOH (100:0, 100:1, 100:5, 90:10, 1:1, and 0:100, each 2 l). The active fraction (90:10) was concentrated in vacuo to remove organic solvent. Finally, the concentrated material (1.6 g) was applied to an HPLC (Chromatorex C8 SPS100-5HE, 20 i.d. × 250 mm) with an isocratic solvent system of 70% acetonitrile-water with 0.1% trifluoroacetic acid solution at a flow rate of 10 ml/min to give two new compounds, trammines A and B (70.7 mg; retention time 48–52 min; 1) and the molecular formula of 1 was elucidated as C28H41NO6 by HR-ESI-MS data ([M–H]− m/z 486.2856: calculated for C28H41NO6, 486.2857), requiring nine degrees of unsaturation. All connections for 1H and 13C in 1 were elucidated by HSQC study (Table 1). The NMR data measured in DMSO-d6 and the molecular mixture of MeOH-H2O (20:80, 50:50, 70:30, 80:20, 90:10, and 100:0; each 2 l). The 80:20 fraction was concentrated to dryness to afford a crude extract (33 g). The extract was chromatographed on a silica gel column and eluted stepwise with a mixture of CHCl3 - MeOH (100:0, 100:1, 100:5, 90:10, 1:1, and 0:100, each 2 l). The active fraction (90:10) was concentrated in vacuo to remove organic solvent. Finally, the concentrated material (1.6 g) was applied to an HPLC (Chromatorex C8 SPS100-5HE, 20 i.d. × 250 mm) with an isocratic solvent system of 70% acetonitrile-water with 0.1% trifluoroacetic acid solution at a flow rate of 10 ml/min to give two new compounds, trammines A (1.15 mg; retention time 64–82 min; 1) and B (70.7 mg; retention time 48–52 min; 2) in Figs. 1a and b.

**Structure Elucidation of 1 and 2**

The molecular formula of 1 was elucidated as C28H41NO6 by HR-ESI-MS data ([M–H]− m/z 486.2856: calculated for C28H41NO6, 486.2857), requiring nine degrees of unsaturation. All connections for 1H and 13C in 1 were elucidated by HSQC study (Table 1). The NMR data measured in DMSO-d6 and the molecular
Table 2 NMR Spectroscopic Data for Traminine B (2) in DMSO-<i>d</i><sub>6</sub>

| Position | δ<sub>C</sub> | mult. | δ<sub>HMBC</sub> (int., mult., J in Hz) | HMBC |
|----------|-------------|-------|--------------------------------------|------|
| 1        | 197.0       | C     |                                      | C-1, C-3, C-4, C-17 |
| 2        | 39.5        | CH    | 3.51 (1H, qt, J = 7.2, 7.8)          | C-1, C-2, C-4, C-5, C-17 |
| 3        | 35.7        | CH<sub>2</sub> | 2.01 (1H, ddd, J = 15.0, 7.8, 7.2) | C-2, C-3, C-6 |
| 4        | 124.7       | CH    | 2.36 (1H, ddd, J = 15.0, 7.8, 7.2)  | C-1, C-2, C-4, C-5, C-17 |
| 5        | 136.3       | CH    | 5.43 (1H, dt, J = 15.6, 7.2)        | C-2, C-3, C-6 |
| 6        | 131.4       | C     |                                      | C-3, C-6, C-7, C-18 |
| 7        | 136.9       | CH    | 5.13 (1H, d, J = 9.0)               | C-5, C-8, C-9, C-18, C-19 |
| 8        | 30.4        | CH    | 2.63 (1H, m)                        | C-6, C-7, C-9, C-10, C-19 |
| 9        | 47.2        | CH<sub>2</sub> | 1.94 (2H, m) | C-7, C-8, C-10, C-11, C-19, C-20 |
| 10       | 134.1       | C     |                                      | C-9, C-12, C-13, C-20 |
| 11       | 126.1       | CH    | 5.71 (1H, d, J = 10.8)              | C-9, C-12, C-13, C-20 |
| 12       | 126.9       | CH    | 6.17 (1H, dd, J = 15.2, 10.8)       | C-10, C-11, C-14 |
| 13       | 132.0       | CH    | 5.51 (1H, dt, J = 15.2, 7.3)        | C-11, C-14, C-15 |
| 14       | 34.4        | CH<sub>2</sub> | 2.00 (2H, td, J = 7.5, 7.3) | C-12, C-13, C-15 |
| 15       | 22.2        | CH<sub>2</sub> | 1.33 (2H, qt, J = 7.2, 7.5) | C-13, C-14, C-16 |
| 16       | 13.6        | CH<sub>3</sub> | 0.83 (3H, t, J = 7.2) | C-14, C-15 |
| 17       | 16.1        | CH<sub>2</sub> | 0.97 (3H, d, J = 7.2) | C-1, C-2, C-3 |
| 18       | 12.4        | CH<sub>3</sub> | 1.63 (3H, s)<sup>a</sup> | C-5, C-6, C-7 |
| 19       | 20.4        | CH<sub>3</sub> | 0.84 (3H, d, J = 7.2) | C-8, C-9 |
| 20       | 16.3        | CH<sub>3</sub> | 1.63 (3H, s)<sup>a</sup> | C-9, C-10, C-11 |
| 2'        | 167.0       | C     |                                      | C-2', C-3', C-4' |
| 3'        | 105.1       | C     |                                      | C-5', C-7', C-8' |
| 4'        | 188.6       | C     |                                      | C-4', C-5', C-6', C-8' |
| 5'        | 67.7        | CH    | 4.60 (1H, d, J = 3.3)               | C-2', C-3', C-4' |
| 6'        | 67.0        | CH    | 4.51 (1H, dd, J = 3.3, 3.6)         | C-5', C-7', C-8' |
| 7'        | 46.0        | CH<sub>2</sub> | 2.27 (1H, d, J = 16.8) | C-4', C-5', C-6', C-8' |
| 8'        | 172.0       | C     | 2.95 (1H, dd, J = 16.8, 3.6)        | C-4', C-5', C-6', C-8' |

Note: <sup>a</sup>Overlapped.

Data were collected at 600 MHz for <sup>1</sup>H and 150 MHz for <sup>13</sup>C.

The similarity in both physicochemical properties and NMR spectrum between 1 and 2 strongly suggested that 2 is a new analog of 1. The molecular formula of 2 was elucidated as <i>C<sub>27</sub>H<sub>38</sub>NO<sub>5</sub></i> by HR-ESI-MS data ([M + H]<sup>+</sup>, m/z 456.2728: calculated for <i>C<sub>27</sub>H<sub>38</sub>NO<sub>5</sub>, 456.2750), requiring ten degrees of unsaturation. The <sup>1</sup>H and <sup>13</sup>C NMR spectra of 2 is listed in Table 2. NMR spectra of 2 lacked a methoxy group and changed the carbon chemical shifts at C-2' (δ<sub>C</sub> 167.0), C-4' (δ<sub>C</sub> 188.6), and C-7' (δ<sub>C</sub> 46.0), the tetratic acid moiety of 1. The HMBC experiments (Fig. 2b) and six remaining degrees of unsaturation gave the following results; the proposed fused ring core structure containing tetratic acid moiety was confirmed by the key HMBC correlations: from H-5' (δ<sub>H</sub> 4.60) to C-2', C-3' (δ<sub>C</sub> 105.1), and C-4'; from H-6' (δ<sub>H</sub> 4.51) to C-7' and C-8' (δ<sub>C</sub> 172.0); from H-2' (δ<sub>H</sub> 2.27 and 2.95) to C-5' (δ<sub>C</sub> 67.7), C-6' (δ<sub>C</sub> 67.0), and C-8'; and from H-7' to N-1' (δ<sub>H</sub> 221.7, Supplementary Fig. S12) position by <sup>1</sup>H-<sup>13</sup>C HMBC correlation. <sup>1</sup>H-<sup>13</sup>C COSY of 2 indicated alignments from H-5' to H-2' as shown in Fig. 2b. The chemical shifts of the side chain, both the <sup>1</sup>H-<sup>13</sup>C COSY and HMBC
correlations (from C-1 to C-20) is almost the same as those of 1. The geometry of double bonds at C-4 and C-5, C-12 and C-13, C-6 and C-7, and C-10 and C-11 were also determined to be of the (E)-configuration on the basis of the large vicinal coupling constants ($J_{4,5} = 15.6$ Hz and $J_{12,13} = 15.2$ Hz, respectively) and ROESY experiment (Supplementary Fig. S13). Therefore, the planar structure of 2 was elucidated as shown in Fig. 1b and we designated 2 to be tramine B.

Compound 1 was a novel compound that, unlike harzicnic acid and JBIR-22, contained a 3-hydroxyglutamatic group bound to tetracitic acid (Izumiwaka et al., 2010; Kawada et al., 2004). Also, it contained a polyketide side chain with a distinctive structure. Interestingly, this polyketide structure has never been found previously in natural products. Compound 2 had a structure similar to that of 1, but with a distinctive novel cyclic tacitic acid structure. Although 2 was a little similar to talarotoxin and burnetricic acids (Ishii et al., 1995; Li et al., 2019), its structure is very rare among natural products. The structure of the polyketide side chain in 2 was also distinctive. When evaluating the biological activity, care was taken about the stability of 2. The purity of 2 used for the evaluation of biological activity was tested in the Supplementary Fig. S14.

**Evaluation of MIC Values of 1 and 2 Against Yeasts**

We measured the MICs of 1 and 2 against wild type and multidrug-sensitive S. cerevisiae (Table 3). Compounds 1 and 2 did not demonstrate strong inhibition against the wild type yeast BY4741 grown on YPD medium. It is interesting that 1 and 2 showed strong inhibition against the multidrug-sensitive yeast grown on YPG medium. The selectivity of 1 and 2 was > 64 and > 128 times, respectively. Compounds 1 and 2 did not show growth inhibition against the wild type yeast, it is strongly suggested that the multidrug-sensitive budding yeast is a useful tool to find new growth inhibition compounds. Therefore, we assumed that the target of 1 and 2 is any one of the mitochondrial functions, such as the electron transfer system. Evaluation of the growth inhibition of the yeast by 1 and 2 suggested that they may be inhibitors of mitochondrial functions because these compounds inhibited the growth of the multidrug-sensitive budding yeast S. cerevisiae 12geneA0HSR-iERG6 grown on YPG agar medium but did not on YPD agar medium.

**Effects of 1 and 2 on the ATP Synthesis in Yeast Mitochondria**

Since yeast glycerol metabolism depends on oxidative phosphorylation, the increased sensitivity to 1 and 2 in glycerol medium strongly suggests that they target mitochondrial machineries presiding over ATP production via oxidative phosphorylation. Based on the assay monitoring overall ADP-uptake/ATP-release reactions with isolated S. cerevisiae mitochondria (Unten et al., 2019), we evaluated the effects of 1 and 2 on ATP synthesis via oxidative phosphorylation. This assay system generally enables us to determine potential inhibitors for any of the mitochondrial proteins presiding over ATP synthesis via oxidative phosphorylation, such as respiratory enzymes (type-2 NADH dehydrogenase [NDH-2], and complexes II-IV), F$_{1}$F$_{0}$-ATPase (complex V), and mitochondrial transporters (VDAC, ADP/ATP carrier, and phosphate carrier). As listed in Table 4, 1 and 2 inhibited overall ADP-uptake/ATP-release reactions with IC$_{50}$ values of 120 and 3.8 nmol/mg of protein respectively. We confirmed, as a control, that carboxyatractyloside (CATR, an inhibitor of ADP/ATP carrier) and antimycin A (an inhibitor of complex III) also inhibited the reaction (Table 4).

---

**Table 3 MIC Values of 1 and 2 Against Yeast**

| Compound | BY4741 | 12geneA0HSR-iERG6 |
|----------|--------|--------------------|
|          | YPD   | YPG                | YPD   | YPG   |
| 1        | >128  | >128               | >128  | 2     |
| 2        | >128  | 32                 | >128  | 1     |

**Table 4 The IC$_{50}$ Values of the Test Compounds in Overall ADP-Uptake/ATP-Release Reactions with Isolated S. cerevisiae Mitochondria**

| Compound | IC$_{50}$ (nmol/mg of proteins) |
|----------|---------------------------------|
| tramine A (1) | 120 ± 10                      |
| tramine B (2) | 3.8 ± 0.6                      |
| CATR       | 0.13 ± 0.01                    |
| antimycin A | 0.10 ± 0.01                    |

Note: The IC$_{50}$ value is the concentration (nmol/mg of proteins) needed to reduce the control ATP efflux from yeast mitochondria by 50%. The average ATP efflux in the absence of inhibitor was 0.16 ± 0.01 μmol ATP/min/mg of proteins. Values are means ± standard error.
Identification of the Target Enzymes of 1 and 2

As the mitochondrial respiratory enzymes provide the major driving force for ADP uptake and ATP synthesis by generating electrochemical proton gradient, we examined the effects of 1 and 2 on each respiratory enzymes and F$_{0}$F$_{1}$-ATPase (Fig. 3). The concentrations of 1 and 2 were set to 360 and 11 nmol/mg of proteins, respectively, which are equivalent to 3 × IC$_{50}$ obtained in the ADP-uptake/ATP-release reactions described above. As shown in Figs. 3a and 3d, both compounds exhibit no inhibition of NDH-2 and complex IV activities. Compound 2 inhibited succinate-cyt. c oxidoreductase (covering complexes II and III) and NADH-cyt. c oxidoreductase (covering NDH-2 and complex III) activities by approximately 70% (Fig. 3b and c). As both assays include electron flux through complex III, these results indicate that the potential target of 2 is respiratory complex III. On the other hand, 1 was found to inhibit ATP hydrolysis (Fig. 3e) as well as succinate- and NADH-cyt. c oxidoreductase activities (Fig. 3b and c). We note that, at higher concentration (40 × IC$_{50}$), 2 also inhibited ATP hydrolysis by around 30% (data not shown), but this effect was negligible at the concentration range equivalent to 1–5 × IC$_{50}$ that was determined in the ADP-uptake/ATP-release assay. Altogether, inhibition of oxidative phosphorylation by 1 is attributed to the dual inhibition of complex III (ubiquinol-cyt. c oxidoreductase) and F$_{0}$F$_{1}$-ATPase with IC$_{50}$ values of 1.100 and 200 nmol/mg of proteins, respectively; whereas that by 2 is due to the inhibition of complex III with IC$_{50}$ value of 6.8 nmol/mg of proteins.

Identification of the Binding Site of 2 in Complex III

Complex III contains two reaction sites for ubiquinone/ubiquinol: one is proximal to the matrix side (Q$_{i}$ site), where an electron is transferred from cytochrome b (containing hemes $b_{i}$ and $b_{h}$) to ubiquinone, and the other is proximal to the intermembrane space (Q$_{o}$ site), where an electron is transferred from ubiquinol to Rieske iron-sulfur cluster, followed by the reduction of cytochrome c$_{1}$ (containing heme c$_{1}$). Complex III inhibitors are generally classified into two groups called ‘Q$_{i}$ site’ and ‘Q$_{o}$ site’ inhibitors, which are represented by antimycin A and myxothiazol, respectively (Crofts, 2004; Trumpower, 1990). To identify the binding site of 2 in complex III (Q$_{o}$ site or Q$_{i}$ site), we examined the effects of 2 on the reduction kinetics of cytochromes b and c hemes using bovine heart SMPs. Prior to the analysis, we confirmed that 2 inhibits complex III in SMPs with IC$_{50}$ value of 9.5 nmol/mg of proteins.

It is a well-known phenomenon that cytochrome b hemes are immediately reduced by the addition of respiratory substrate in the presence of Q$_{i}$ or Q$_{o}$ site inhibitor. In agreement of previous studies (Link et al., 1993; Matsuno-Yagi & Hatefi, 1996; Trumpower, 1990), only the simultaneous addition of Q$_{i}$ and Q$_{o}$ site inhibitors (antimycin A and myxothiazol, respectively) led to the suppression of the b hemes reduction (Fig. 4a, traces a–c and e). The addition of 2 alone did not affect the reduction level of the b hemes (trace d), while the simultaneous addition of 2 and antimycin A markedly inhibited the b hemes reduction (trace f). We noted that a slow increase in the b heme reduction was observed in the presence of antimycin A and myxothiazol, but not in the presence of antimycin A and 2 (traces e and f). A pair of myxothiazol and 2 did not suppress the b hemes reduction (trace g). Next, we investigated the effect of inhibitors on the c$_{1}$ heme reduction. Antimycin A did not affect the reduction level of the c$_{1}$ heme, whereas myxothiazol significantly reduced the reduction level (Fig. 4b, traces a–c) (Link et al., 1993; Matsuno-Yagi & Hatefi, 1996; Trumpower, 1990). The extent of suppression of the c$_{1}$ heme reduction by 2 was less than that by myxothiazol; however, once reduced, c$_{1}$ heme slowly reoxidized (trace d). Although we have no definite explanation of this phenomenon at present, this result suggests that structure of the c$_{1}$ heme environment could be destabilized by 2. Taking all these results together, we conclude that 2 binds to the Q$_{o}$ site in complex III, which is a slightly different manner from that of myxothiazol.

![Fig. 3](image.png) Effects of traminines A (1) and B (2) on the respiratory enzyme activities. The effects of 1 and 2 on the activity of each respiratory complex. NADH-UQ$_{i}$ oxidoreductase (NDH-2, a), succinate-cyt. c oxidoreductase (complexes II–III, b), NADH-cyt. c oxidoreductase (NDH-2–complex III, c), and cyt.-c oxidase (complex IV, d) activities in addition to ATP hydrolysis (FoF$_{1}$-ATPase, e) were measured in the presence of 1 and 2. In these assays, the concentration of 1 and 2 were set to 360 and 11 nmol/mg of proteins, respectively, which are equivalent to 3 × IC$_{50}$ obtained in the ADP-uptake/ATP-release reactions. Values show means ± SEM (n = 3–4).
Fig. 4 Effects of traminine B (2) on the reduction of cytochrome b and c hemes of complex III in bovine SMPs. (a) The reduction of cytochrome b hemes was monitored at the wavelength pair of 563 and 575 nm. SMPs (1.2 mg/ml) were incubated with various inhibitors for 4 min before the addition of sodium succinate (5 mM). The complete reduction of the b hemes was achieved by the addition of Na2S2O4. Traces are as follows: a, control; b, antimycin A (AA, 2 μM); c, myxothiazol (MX, 4 μM); d, traminine B (2, 23 μM); e, antimycin A and myxothiazol; f, antimycin A and 2; g, myxothiazol and 2. (b) The reduction of cytochrome c1 heme was monitored at the wavelength pair of 539 and 553 nm. Traces are as follows: a, control; b, antimycin A; c, myxothiazol; d, 2. Data are representative of three independent measurements. X axis is time, Y axis is ΔAbs.

Conclusion
Naturally occurring tetramic acids have attracted a great deal of attention for their broad spectrum of biological activities (Mo et al., 2014). Fusaramin (Sakai et al., 2019), equisetin (König et al., 1993), and equisetin-like compound TA-289 (Quek et al., 2013) have been reported to interfere with mitochondrial oxidative phosphorylation; however, to the best of our knowledge, there was no report on their target protein(s) in mitochondria. Throughout bioassay-guided screening with multidrug-sensitive S. cerevisiae Δ0HSR-iERG6 strain, we here isolated two new tetramic acid derivatives traminines A (1) and its intramolecular N-acylation product traminine B (2) from the culture broth of a fungal strain Fusarium concentricum FKI-7550. Both compounds were found to inhibit ATP production via oxidative phosphorylation in S. cerevisiae mitochondria. Detailed biochemical characterization revealed that 1 elicits dual inhibition of respiratory complex III and F1F0-ATPase, whereas 2 inhibits solely complex III (at Qo site). Further structural modifications of these compounds would contribute greatly to the development of new types of pharmaceutical and agrochemical lead compounds.

Acknowledgments
We thank Dr. K. Nagai and Ms. N. Sato (School of Pharmacy, Kitasato University) for various instrumental analyses. This article is dedicated to the fond memory of Professor Arnold L. Demain.

Supplementary Material
Supplementary material is available online at JIMB (www.academic.oup.com/jimb).

Author Contributions
Kazuro Shiomi, Hideto Miyoshi, Yukihiro Asami and Satoshi Ōmura designed the research. Katsuyuki Sakai, Aoi Kimishima
and Masato Iwatsuki isolated and analyzed the compounds. Yufu Unten, Masatoshi Murai and Hideto Miyoshi evaluated the biological activities. Kenichi Nonaka isolated and maintained the fungal strain. Takumi Chinen and Takeo Usui established the yeast strain. Katsuyuki Sakai, Kazunari Sakai, Yufu Unten, Masatoshi Murai, Hideto Miyoshi and Yukihiro Asami wrote the paper. All authors approved the final manuscript.

**Funding**

This study was partially supported by the Platform Project for Supporting Drug Discovery and Life Science Research (Basis for Supporting Innovative Drug Discovery and Life Science Research [BINDS]) from the Japan Agency for Medical Research and Development (AMED) under Grant Number JP20am0101096. Moreover, this research was partly supported by AMED under Grant Number 20am0101047 as well. The study was supported by The Public Foundation of Elizabeth Arnold–Fuji, Japan.

**Conflict of Interest**

No potential conflict of interest was reported by the authors.

**References**

Bald, D., Villellas, C., Lu, P., & Koul, A. (2017). Targeting energy metabolism in *Mycobacterium tuberculosis*, a new paradigm in antimycobacterial drug discovery. mbio, 8(2), e00272–17.

Chinen, T., Nagumo, Y., & Usui, T. (2014). Construction of a genetic library of highly purified mitochondrial yeast strain by disruption of the drug efflux system and conditional repression of the membrane barrier system. *The Journal of General and Applied Microbiology*, 60(4), 160–162.

Crofts, A. R. (2004). The cytochrome b, c complex: function in the context of structure. *Annual Review Physiology*, 66, 689–733.

Fukuda, T., Sudo, Y., Tsuchiya, Y., Okuda, T., Matsuura, N., Motojima, A., Oikawa, T., & Igarashi, Y. (2015). Tolypoalbin, a new tetracatic acid from *Tolypocladium album* TAMA 479. *The Journal of Antibiotics*, 68(6), 399–402.

Glick, B. S. & Pon, L. A. (1995). Isolation of highly purified mitochondria from *Saccharomyces cerevisiae*. Methods in Enzymology, 260, 213–223.

Izumikawa, M., Hashimoto, J., Hirokawa, T., Sugimoto, S., Kato, T., Takagi, M., & Shin-ya, K. (2010). JBI-R 22, an inhibitor for protein-protein interaction of the homodimer of proteasome assembly factor 3. *Journal of General and Applied Microbiology*, 24(3), 232–237.

Ishii, K., Itoh, T., Kobayashi, K., Horie, Y., & Ueno, Y. (1995). Isolation and characterization of a cytotoxic metabolite of *Talaromyces bacillisporus*. *Applied and Environmental Microbiology*, 61(3), 941–943.

Kemami-Wangun, H. V. & Hertweck, C. (2007). Epicoccarinines A, B and epiypyrindone: tetracatic acids and pyridone alkaloids from an *Epicoccum* sp. associated with the tree fungus *Pholiota squarrosa*. *Organic & Biomolecular Chemistry*, 5(11), 1702–1705.

Kita, K., Shiomi, K., & Ōmura, S. (2007). Advances in drug discovery and biochemical studies. *Trends in Parasitology*, 23(5), 223–229.

Kawada, M., Yoshimoto, Y., Kumagai, H., Someno, T., Momose, I., Kawamura, N., Isshiki, K., & Ikeda, D. (2004). PP2A inhibitors, harzicianic acid and related compounds produced by fungus strain F-1531. *The Journal of Antibiotics*, 57(3), 243–246.

Kelsa, G. F., Porteous, C. M., Coulter, C. V., Hughes, G., Porteous, W. K., Ledgerwood, E. C., Smith, R. A. J, & Murphy, M. P. (2001). Selective targeting of a redox-active ubiquinone to mitochondria within cells: antioxidant and antiapoptotic properties. *Journal of Biological Chemistry*, 276(7), 4588–4596.

König, T., Kapos, A., & Sarkadi, B. (1993). Effects of equisetin on rat liver mitochondria: evidence for inhibition of substrate anion carriers of the inner membrane. *Journal of Bioenergetics and Biomembranes*, 25(5), 537–545.

Li, H., Gilchrist, C. L. M., Lacey, H. J., Crombie, A., Vuong, D., Pitt, J. I., Lacey, E., Chooi, Y. H., & Piggott, A. M. (2019). Discovery and heterologous biosynthesis of the burnettramic acids: rare PKS-NRPS-derived balaamphillic pyrrolizidinediones from an Australian fungus, *Aspergillus burnetti*. *Organic Letters*, 21(5), 1287–1291.

Link, T. A., Haase, U., Brandt, U., & von Jagow, G. (1993). What information do inhibitors provide about the structure of the hydroquinone oxidation site of ubiquinone: cytochrome c oxidoreductase? *Journal of Bioenergetics and Biomembranes*, 25(3), 221–232.

Mo, X., Li, Q., & Ju, J. (2014). Naturally occurring tetramic acid products: isolation, structure elucidation and biological activity. *RSC Advances*, 4(92), 50566–50593.

Matsuno-Yagi, A. & Hatefi, Y. (1996). Ubiquinol-cytochrome c oxidoreductase. The redox reactions of the bis-heme cytochrome b in ubiquinone-sufficient and ubiquinone-deficient systems. *Journal of Biological Chemistry*, 271(11), 6164–6171.

Matsuno-Yagi, A. & Hatefi, Y. (1985). Studies on the mechanism of oxidative phosphorylation. *Journal of Biological Chemistry*, 260(27), 14424–14427.

Newman, D. J. & Cragg, G. M. (2020). Natural products as sources of new drugs over the nearly four decades from 01/1981 to 09/2019. *Journal of Natural Products*, 83(3), 770–803.

Ömura, S., Asami, Y., & Crump, A. (2018). Stauroporine: new lease of life for parent compound of today’s novel and highly successful anti-cancer drugs. *The Journal of Antibiotics*, 71(8), 688–701.

Quek, N. C., Matthews, J. H., Bloor, S. J., Jones, D. A., Bircham, P. W., Heathcott, R. W., & Atkinson, P. H. (2013). The novel equisetin-like compound, TA-289, causes aberrant mitochondrial morphology which is independent of the production of reactive oxygen species in *Saccharomyces cerevisiae*. *Molecular BioSystems*, 9(8), 2125–2133.

Suga, T., Asami, Y., Hashimoto, S., Nonaka, K., Iwatsuki, K., Nakashima, T., Sugahara, R., Shiotsuki, T., Yamamoto, T., Shinohara, Y., Ichimaru, N., Murai, M., Miyoshi, H., Ömura, S., & Shiomi, K. (2015). Ascosteroside C, a new mitochondrial respiration inhibitor discovered by pesticidal screening using recombinant *Saccharomyces cerevisiae*. *The Journal of Antibiotics*, 68(10), 649–652.

Sakai, K., Unten, Y., Iwatsuki, M., Matsuo, H., Fukasawa, W., Hirose, T., Chinen, T., Nonaka, K., Nakashima, T., Sunazuka, T., Usui, T., Murai, M., Miyoshi, H., Ömura, S., & Shiomi, K. (2019). Fusaramin, an antimitochondrial compound produced by *Aspergillus burnetti* sp., discovered using multidrug-sensitive yeast strain by disruption of the drug efflux system and conditional repression of the membrane barrier system. *Journal of Antibiotics*, 72(9), 645–652.

Shang, Z., Li, L., Espósito, B. P., Salim, A. A., Khalil, Z. G., Quezada, M., Bernhardt, P. V., & Capon, R. J. (2015). New PKS-NRPS tetramic acids and pyridinone from an Australian marine-derived fungus, *Chaunopycnis visiae* sp., discovered using multidrug-sensitive yeast strain. *Journal of General and Applied Microbiology*, 61(3), 260, 261–265.

Trumpower, B. L. (1990). The protonmotive Q cycle. Energy transduction by coupling of proton translocation to electron transfer by the cytochrome bc$_1$ complex. *Journal of Biological Chemistry*, 265(20), 11409–11412.
type compounds specifically bind to voltage-dependent anion channel 1 in *Saccharomyces cerevisiae* mitochondria. *Biochemistry*, 58(8), 1141–1154.

Weinberg, S. & Chandel, N. S. (2015). Targeting mitochondria metabolism for cancer therapy. *Nature Chemical Biology*, 11(1), 9–15.

Watanabe, Y., Suga, T., Narusawa, S., Iwatsuki, M., Nonaka, K., Nakashima, T., Shinohara, Y., Shiotsuki, T., Ichimaru, N., Miyoshi, H., Asami, Y., Ōmura, S., & Shiomi, K. (2017). Decatamariic acid, a new mitochondrial respiration inhibitor discovered by pesticidal screening using drug-sensitive *Saccharomyces cerevisiae*. *The Journal of Antibiotics*, 70(4), 395–399.

Xu, W., Cai, X., Jung, M. E., & Tang, Y. (2010). Analysis of intact and dissected fungal polyketide synthase-nonribosomal peptide synthetase in vitro and in *Saccharomyces cerevisiae*. *Journal of American Chemical Society*, 132(39), 13604–13607.

Yabunaka, H., Kenmochi, A., Nakatogawa, Y., Sakamoto, K., & Miyoshi, H. (2002). Hybrid ubiquinone: novel inhibitor of mitochondrial complex I. *Biochimica et Biophysica Acta*, 1556(2-3), 106–112.