Available online at www.sciencedirect.com
SCIENCE DIRECT®
PHYTOCHEMISTRY
Phytochemistry 65 (2004) 1291–1298
www.elsevier.com/locate/phytochem

Antibacterial diterpenes and their fatty acid conjugates from rice leaves

Yoshiki Kono [a,*], Aya Kojima [a], Rieko Nagai [b], Manabu Watanabe [b], Takahiro Kawashima [b], Tsugu Onizawa [b], Tohru Teraoka [b], Minoru Watanab [b], Hiroyuki Koshino [c], Jun Uzawa [c], Yoshikatsu Suzuki [c], Akira Sakurai [c]

[a] *School of Agriculture, University of Ibaraki, Ami, Ibaraki 300-0393, Japan*
[b] *Tokyo University of Agriculture and Technology, Fuchu-shi, Tokyo 183-8509, Japan*
[c] *Riken (The Institute of Physical and Chemical Research), Hirosawa 2-1, Wako-shi, Saitama 351-0198, Japan*

Received 14 November 2003; received in revised form 25 February 2004
Available online 23 April 2004

Abstract

Six structurally oryzalide-related compounds, oryzadione (**1**), **2**, **3**, **4**, **5** and **6**, were isolated from a neutral fraction of the extract of healthy leaves using a bacterial leaf blight-resistant cultivar of a rice plant, "Norin-27", as a group of antimicrobial substances. Their structures were determined by spectroscopic studies to be kaurane analogues and kaurane analogues conjugated with fatty acids, i.e., **1**: *ent*-15,16-epoxy-kauran-2,3-dione (enol form: *ent*-15,16-epoxy-2-hydroxy-kauran-1-en-3-one), **2**: *ent*-15,16-epoxy-3β-hydroxy-kauran-2-one, **3**: *ent*-15,16-epoxy-3-oxa-kauran-2-one, **4**: *ent*-15,16-epoxy-3β-myristoyloxy-kauran-2-one, **5**: *ent*-15,16-epoxy-3α-palmitoyloxy-kauran-2-one, and **6**: *ent*-15,16-epoxy-2β-palmitoyloxy-kauran-2-one.

Keywords: *Oryza sativa*; Gramineae; Rice plant; Resistance against *Xanthomonas campestris* pv. *oryzae*; Antibacterial diterpene; *ent*-Kaurane analogue; Myristoyloxy-kaurane; Palmitoyloxy-kaurane

1. Introduction

The induction of resistance in rice plants against a bacterial leaf blight was investigated and the lesion enlargement by a compatible strain of the causal bacterium, *Xanthomonas campestris* pv. *oryzae*, was markedly inhibited as a result of resistance-induction by inoculation of incompatible strains of the same bacterium (Watanabe et al., 1976). In leaves inoculated with the incompatible strains, EtOAc-extractable acidic antibacterial substances were detected as early as 24 h post-inoculation, increased rapidly to the maximum level within 3–5 days, and remained constant until 14 days after inoculation (Nakanishi and Watanabe, 1977a).

An abundant production of EtOAc-extractable acidic antibacterial substances in the resistance-induced leaves was observed, and they were also found in healthy rice leaves (Nakanishi and Watanabe, 1977a,b). In an attempt to isolate these compounds, we isolated and determined the structure of the antibacterial diterpene, oryzalide A (*ent*-15,16-epoxy-1α-hydroxy-2-oxa-kauran-3-one), from the EtOAc extractable acidic fraction obtained from healthy leaves of "Norin-27", a cultivar of *Oryza sativa* that is resistant against *X. campestris* pv. *oryzae* T7174 (Watanabe et al., 1990; Kono et al., 1991). We also succeeded in isolating three acidic antibacterial compounds, oryzalide B, oryzalic acid A (Kono et al., 1991) and oryzalic acid B, and three related compounds A, B and C (Watanabe et al., 1992) as a group of antibacterial diterpenes, and their structures were determined to be *ent*-kaurane or *ent*-kaurene analogues.

The GC-SIM analysis data of Norin-27 and Rantai emas leaves inoculated with an incompatible strain of *X. campestris* pv. *oryzae* indicated a greater accumulation of oryzalides and oryzalic acids, especially in the lesion area than healthy leaves, and mechanical injury by a needle-bundle also increased the total amounts

* Corresponding author. Tel./fax: +81-298-88-8686.
E-mail address: ykono@mx.ibaraki.ac.jp (Y. Kono).

0031-9422/$ - see front matter © 2004 Elsevier Ltd. All rights reserved.
doi:10.1016/j.phytochem.2004.03.016

(Watanabe et al., 1996). The results of quantitative analysis suggested that these antibacterial compounds might act to protect the rice leaves from bacterial invasion, and thus might be viewed as one of the inhibitins.

In a continuation of the work, an antibacterial oryzalide-related compound from the neutral fraction of the leaf extract was investigated to obtain useful compounds for investigating the biosynthetic pathways of oryzalides. As a result, oryzadione (**1**), **2**, **3**, **4**, **5** and **6** were isolated from the neutral fraction and their structures were determined. In this paper, we report the isolation procedures and the results of structural determination of these six compounds.

2. Results and discussion

2.1. Isolation of *1, 2, 3, 4, 5* and *6* from leaves of rice plant

Flag leaves (the uppermost leaf at the ripening stage; 5.5 kg) of Norin-27 were extracted with 80% aq MeOH and the filtrate was concentrated in vacuo to an aqueous solution. The concentrated aqueous solution was extracted with EtOAc at pH 8.4, the EtOAc extract purified by column chromatography and prep. TLC using color reaction with 5% vanillin-H_2SO_4 (120 °C for 5 min; a pinkish spot) as a method for detecting active compounds to give **1** (3.1 mg), **2** (3.2 mg) and **3** (1.2 mg).

In the other experiment, fresh leaves (1 kg) of ripening stage of Norin-27 were extracted with 80% aq MeOH and concentrated to an aqueous solution as above. The concentrated aqueous solution was dissolved stepwise with H_2O, 20%, 50%, 80% and 100% MeOH, and each solution was successively injected into an ODS column and eluted with 2 l of the same solvent. The MeOH eluate was purified by silica gel column chromatography using GC-SIM for detecting the precursor of oryzalic acid A (see Section 3.4) and the color reaction with 5% vanillin–H_2SO_4, which detected **4** (5.0 mg), **5** (1.5 mg) and **6** (5.0 mg).

2.2. Determination of structures of *1, 2, 3, 4, 5* and *6*

The molecular formula of **1**, **2** and **3** were determined to be $C_{20}H_{28}O_3$, $C_{20}H_{30}O_3$ and $C_{19}H_{28}O_3$ by HREIMS, respectively.

The ^1H and ^{13}C NMR spectral data of **1** (Tables 1 and 2) indicated many common signals which were attributable to four characteristic methyl groups, an epoxy group, and the B- and C-ring moieties as those of ory-

Table 1
Assignments of signals in the proton NMR spectra of oryzadione (**1**), **2**, **3**, **4**, **5** and **6**

	Oryzadione (**1**)	**2**	**3**	**4**	**5**	**6**
1	6.44	2.07/2.69	1.88/2.80	2.12/2.61	2.23/2.51	1.78/2.18
2	5.94 (OH)	–	–	–	–	5.63
3	–	3.89/3.45 (OH)	– (O)	4.92	4.89	–
4	–	–	–	–	–	–
5	1.66	1.49	1.49	1.52	1.47	1.84
6	1.43/1.59	1.34/1.73	1.42/1.52	1.33/1.72	1.43[a]/1.64[a]	1.32/1.56
7	1.61/1.81	1.63/1.82	1.58/1.82	1.61/1.81	1.61/1.80	1.54/1.78
8	–	–	–	–	–	–
9	1.47	1.47	1.27	1.44	1.42	1.43
10	–	–	–	–	–	–
11	1.72 (2H)[a]	1.46/[b]	1.47/1.69	[b]	1.60[a]/1.80[a]	1.44/1.72
12	1.58/1.73	1.57/1.67	1.49/1.58	1.52/1.63	1.68[a]/[b]	1.52/1.67
13	2.17	2.15	2.17	2.13	2.14	2.12
14	1.13/1.49	1.08/1.46	1.12/1.46	1.09/1.43	1.09/1.48	1.08/1.38
15	2.71	2.71	2.70	2.70	2.69	2.69
16	–	–	–	–	–	–
17 Me	1.45	1.44	1.45	1.42	1.43	1.44
18 Me	1.22	1.20	1.44	1.09	0.99	1.20
19 Me	1.13	0.69	1.35	0.84	0.95	1.07
20 Me	1.30	0.96	1.16	0.98	1.20	0.95
1′	–	–	–	–	–	–
2′	–	–	–	2.44	2.41	2.40
3′	–	–	–	1.60	1.65	1.65
4′–11′	–	–	–	1.25–1.30[a]	1.25–1.30[a]	1.25–1.30[a]
12′	–	–	–	1.27[a]	1.25–1.30[a]	1.25–1.30
13′	–	–	–	1.27	1.25–1.30[a]	1.25–1.30
14′	–	–	–	0.87	1.27[a]	1.27[a]
15′	–	–	–	–	1.27	1.27
16′	–	–	–	–	0.88	0.88

[a] Tentative assignment.
[b] Not assigned.

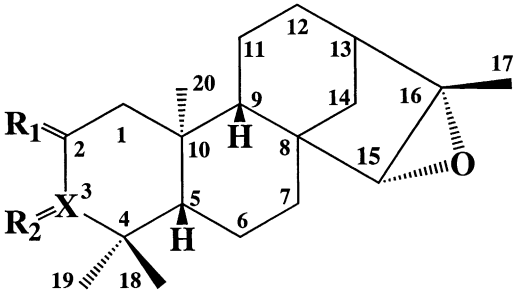
Table 2
Assignments of signals in the carbon-13 NMR spectra of oryzadione (**1**), **2**, **3**, **4**, **5** and **6**

	Oryzadione (**1**)	**2**	**3**	**4**	**5**	**6**
1	128.3	53.4	46.7	54.2	53.2	46.7
2	144.2	210.1	170.3	203.8	206.7	70.7
3	201.0	82.8	– (O)	83.6	82.4	212.1
4	44.0	45.4	85.7	43.5	41.2	46.7
5	53.7	54.1	52.0	55.0	51.4	51.5
6	19.3	19.0	21.5	19.1	19.7	20.4
7	35.1	35.1	34.1	35.1	34.7	33.9
8	43.9	43.8	43.3	43.8	43.7	43.5
9	45.5	50.0	47.2	49.8	50.3	49.1
10	39.9	45.1	37.6	44.5	43.5	38.4
11	18.3	18.2	17.98	18.2	18.4	20.5
12	26.7	26.7	26.4	26.7	26.5	26.6
13	38.9	38.9	38.8	38.9	38.9	38.9
14	32.5	31.6	31.6	31.7	31.4	31.1
15	67.6	67.7	67.3	67.7	67.7	67.4
16	61.3	61.3	61.1	61.2	61.1	61.1
17	14.5	14.4	14.4	14.4	14.4	14.5
18	27.5	29.6	32.6	29.3	26.6	29.1
19	21.5	16.4	24.4	17.4	21.5	19.2
20	22.0	18.5	17.95	18.3	19.4	20.5
1′	–	–	–	173.3	172.9	173.1
2′	–	–	–	34.1	34.1	34.0
3′	–	–	–	25.0	24.9	24.9
4′–11′	–	–	–	29.1–29.7[a]	29.1–29.7[a]	29.1–29.7[a]
12′	–	–	–	31.9	29.1–29.7[a]	29.1–29.7[a]
13′	–	–	–	22.7	29.1–29.7[a]	29.1–29.7[a]
14′	–	–	–	14.1	31.9	31.9
15′	–	–	–	–	22.7	22.7
16′	–	–	–	–	14.1	14.1

[a] Tentative assignment.

zalide A (Kono et al., 1991). The NMR spectral data indicated some differences in chemical shifts of A-ring moiety between **1** and oryzalide A (Kono et al., 1991), i.e., **1**//oryzalide A: δ_H/δ_C 6.44(H-1; CH, s)/128.3 (C-1, d)//5.38 (CH, s)/100.4 (s), 144.2 (C-2, s)//– (–O–), 201.0 (C-3, C═O, s)//179.4 (O–C═O, s) and 44.0 (C-4, s)//42.9* (s, tentative assignment) suggesting an enolic α-diketone group on A-ring moiety of **1**. The UV spectral data of **1** showed an absorption maximum at 273 nm supporting an enolic α-diketone group. From these results, the structural difference between **1** and oryzalide A was explainable by replacing the lactol group (C-1 to C-3) of oryzalide A with an enolic ketone group for **1**. These assumptions were mainly confirmed by HMBC (Fig. 2) and HMQC experiments. Thus, the primary structure of oryzadione (**1**) was determined to be 15,16-epoxy-2-hydroxy-kauran-1-en-3-one (Fig. 1). Though **1** contained an enolic α-diketone group, the EtOAc-extractable acidic fraction was not investigated to isolate the compound.

The UV and IR spectral data of **2** showed absorption maximums at 275 nm (ε185) and 1710 cm^{-1}, respectively, suggesting a ketone group. The ^1H and ^{13}C NMR spectral data indicated some differences in chemical shifts of A-ring moiety between **2** (Tables 1 and 2) and



oryzadione (1):R_1=O, R_2=O, X=C
(enol form): -C_1H=C_2(OH)-, R_2 =O, X=C
2: R_1=O, R_2=H, α-OH, X=C
3: R_1=O, X=O
4: R_1=O, R_2= H, α-O-CO$(CH_2)_{12}CH_3$, X=C
5: R_1=O, R_2=H, β-O-CO$(CH_2)_{14}CH_3$, X=C
6: R_1=H, α-O-CO$(CH_2)_{14}CH_3$, R_2 =O, X=C

Fig. 1. Structures of oryzadione (**1**), **2**, **3**, **4**, **5** and **6**.

compound A (Watanabe et al., 1992), i.e., **2**//compound A: δ_H/δ_C 2.07, 2.69 (H-1; CH_2, m,m)/53.4 (C-1, t)//0.90, 2.22 (H-1; CH_2, m,m)/46.5 (C-1), 210.4 (C-2, C═O, s)// 3.72 (H-2, CH–O, m)/68.7 (C-2) and 3.88 (H-3, s)//2.99 (d) suggesting an α-ketol (2-keto-3-hydroxy) group on A-ring moiety of **2**. From these results, the structural difference between **2** and compound A was explainable by replacing of the hydroxyl at C-2 of compound A with

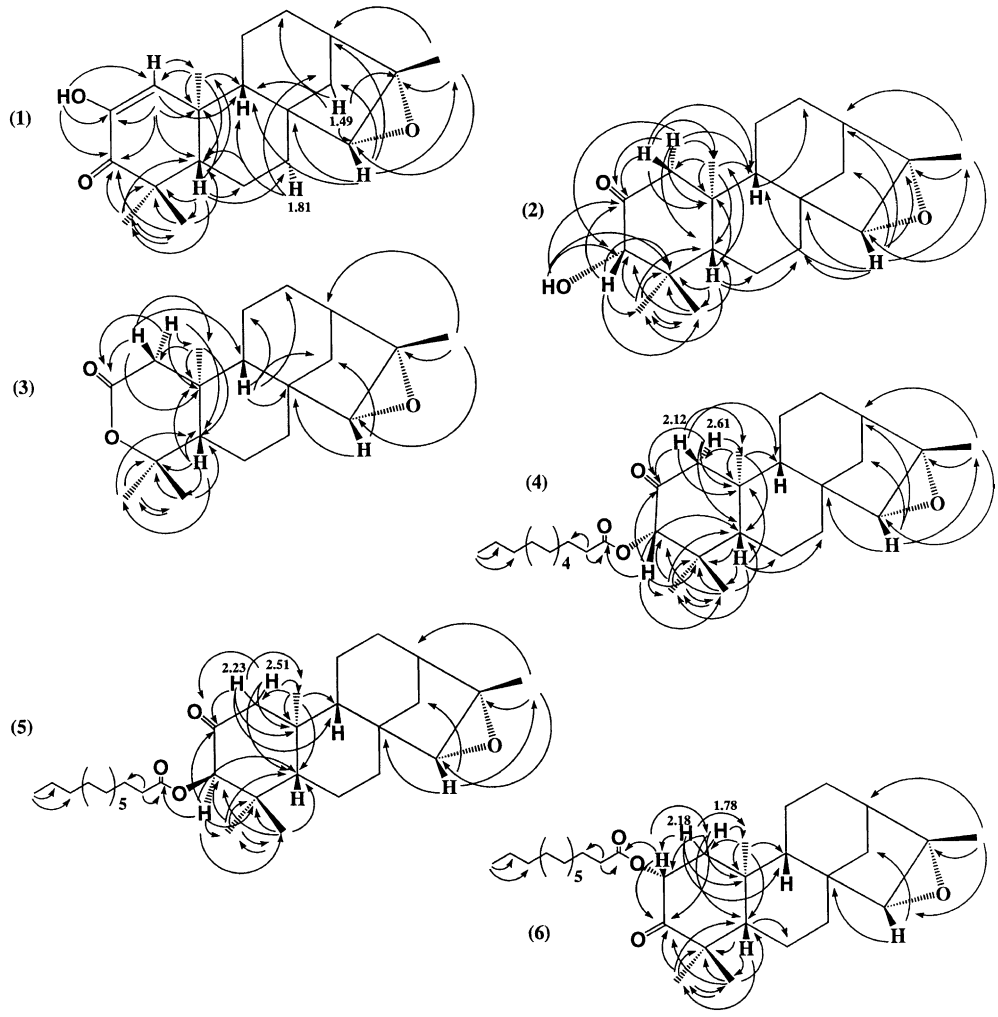


Fig. 2. HMBC correlations used for the structure determination.

carbonyl group for **2**. These assumptions were mainly confirmed by HMBC (Fig. 2) and HMQC experiments. Thus the primary structure of **2** was determined to be 15,16-epoxy-3-hydroxy-kauran-2-one (Fig. 1).

The UV spectrum (MeOH) of **3** showed no absorption maximum above 220 nm. An absorption maximum at 1708 cm^{-1} in the IR spectrum suggested a lactone group. The ^{1}H and ^{13}C NMR spectral data of **3** (Tables 1 and 2) showed some differences from **2**, i.e., δ_C 170.3 (C-2, –O–C=O), 85.7 (C-4, oxygenated quaternary carbon), and methyl protons at δ_H/δ_C 1.44/32.6 (H-18/C-18) and 1.35/24.4 (H-19/C-19), indicating the α-ketol (2-keto-3-hydroxy) group in **2** was replaced with a lactone group between C-2 and C-4 (2-keto-3-oxa) in **3**. These assumptions were mainly confirmed by HMBC (Fig. 2) and HMQC experiments. Thus the primary structure of **3** was determined to be 15,16-epoxy-3-oxa-kauran-2-one as shown in Fig. 1.

The EIMS spectrum of **4** showed $[M]^+$ at m/z 528 (HREIMS calculated for $C_{34}H_{56}O_4$: 528.4175 and observed 528.4147) and $[M-210]^+$ at m/z 318 (base peak above m/z 200; HREIMS calculated for $C_{20}H_{30}O_3$: 318.2193 and observed 318.2165), suggesting a myristoyl ($C_{14}H_{27}O$) derivative of **2**. The ^{1}H and ^{13}C NMR spectral data of **4** (Tables 1 and 2) indicated chemical shifts similar to those of **2** (excepting lower H-3 chemical shift at δ_H 4.92 of **4**; compound **2**, δ_H 3.89). The NMR spectral data of **4** indicated additional signals due to myristoyl moiety, i.e., δ_H/δ_C –/173.3 (1, O–C=O), 2.44/34.1 (2), 1.60/25.0 (3), 1.25–1.30*/29.1–29.7* (4–11, *tentative), 1.27*/31.9 (12), 1.27/22.7 (13) and 0.87/14.1 (14), as shown in Tables 1 and 2. From these results, the structure of **4** was estimated as myristoyl-compound **2**. These assumptions were mainly confirmed by HMBC (Fig. 2) and HMQC experiments. Thus the primary structure of **4** was determined to be 15,16-epoxy-3-myristoyloxy-kauran-2-one (Fig. 1).

The EIMS spectrum of **5** showed $[M]^+$ at m/z 556 (HREIMS calculated for $C_{36}H_{60}O_4$: 556.4488 and observed 556.4494) and $[M-238]^+$ at m/z 318 (base peak

above m/z 200; HREIMS calculated for $C_{20}H_{30}O_3$: 318.2193 and observed 318.2183), suggesting a palmitoyl ($C_{16}H_{31}O$) derivative of **2**. The ^1H and ^{13}C NMR spectral data of **5** (Tables 1 and 2) indicated chemical shifts similar to those of **2** (A- to D-ring moieties; excepting H-3/C-3//H-18/C-18//H-19/C-19//H-20/C-20 chemical shift at δ_H/δ_C 4.89/82.4//0.99/26.6//0.95/21.5// 1.20/19.4 of **5**; **2**, δ_H/δ_C 3.89/82.8//1.20/29.6//0.69/16.4// 0.96/18.5). The NMR spectral data of **5** indicated additional signals due to palmitoyl moiety, i.e., δ_H/δ_C –/ 172.9 (1, O–C=O), 2.41/34.1 (2), 1.65/24.9 (3), 1.25–1.30*/29.1–29.7 (4–13, *tentative), 1.27*/31.9 (14), 1.27/ 22.7 (15) and 0.88/14.1(16), as shown in Tables 1 and 2. From these results, the structure of **5** was estimated as palmitoyl-compound **2**. These assumptions were mainly confirmed by HMBC (Fig. 2) and HMQC experiments. Thus the primary structure of **5** was determined to be 15,16-epoxy-3-palmitoyloxy-kauran-2-one (Fig. 1).

The EIMS spectrum of **6** showed $[M]^+$ at m/z 556 (HREIMS calculated for $C_{36}H_{60}O_4$: 556.4488 and observed 556.4496) and [M-238] $^+$ at m/z 318 (89% of base peak above m/z 200; HREIMS calculated for $C_{20}H_{30}O_3$: 318.2193 and observed 318.2184), suggesting a palmitoyl ($C_{16}H_{31}O$) derivative of **2**. The ^1H and ^{13}C NMR spectral data of **6** (Tables 1 and 2) indicated chemical shifts similar to those of **2** (A- to D-ring moieties). The major difference was observed in H-2/C-2//C-3 chemical shift, i.e., δ_H/δ_C 5.63 (dd)/70.7// 212.1 (**2**, δ_H/δ_C 210.1//3.89/82.8), suggesting a 2-hydroxy-3-oxo- isomer of **2**. The ^1H and ^{13}C NMR spectral data of **6** indicated additional signals due to palmitoyl moiety, i.e., δ_H/δ_C –/173.1 (1, O–C=O), 2.40/34.0 (2), 1.65/24.9 (3), 1.25–1.30*/29.1–29.7 (4–13, *tentative), 1.27*/31.9 (14), 1.27/22.7 (15) and 0.88/ 14.1 (16), as shown in Tables 1 and 2. From these results, the structure of **6** was estimated as palmitoyl-compound **2**. These assumptions were mainly confirmed by HMBC (Fig. 2) and HMQC experiments. Thus the primary structure of **6** was determined to be 15,16-epoxy-2-palmitoyloxy-kauran-3-one (Fig. 1).

The absolute configurations of **1**, **2**, **3**, **4**, **5** and **6** were estimated from the results for related compounds (Watanabe et al., 1990, 1992; Kono et al., 1991) based on consideration of their biosynthetic pathway, and their relative configurations were partially confirmed by NOEdiff spectra (Fig. 3; SELROESY for **2**) to be *ent*-15, 16-epoxy-kauran-2,3-dione (enol form: *ent*-15,16-epoxy-2-hydroxy-kauran-1-en-3-one), *ent*-15,16-epoxy-3β-hydroxy-kauran-2-one, *ent*-15,16-epoxy-3-oxa-kauran-2-one, *ent*-15,16-epoxy-3β-myristoyloxy-kauran-2-one, *ent*-15, 16-epoxy-3α-palmitoyloxy-kauran-2-one and *ent*-15,16-epoxy-2β-palmitoyloxy-kauran-3-one, respectively. The assignments of ^1H and ^{13}C NMR spectral data of **1**, **2**, **3**, **4**, **5** and **6** were achieved using 2D NMR (COSY, HMQC and HMBC) and 1D NMR (INEPT and NOEdiff; SELROESY for **2**) experiments as given in Tables 1 and 2.

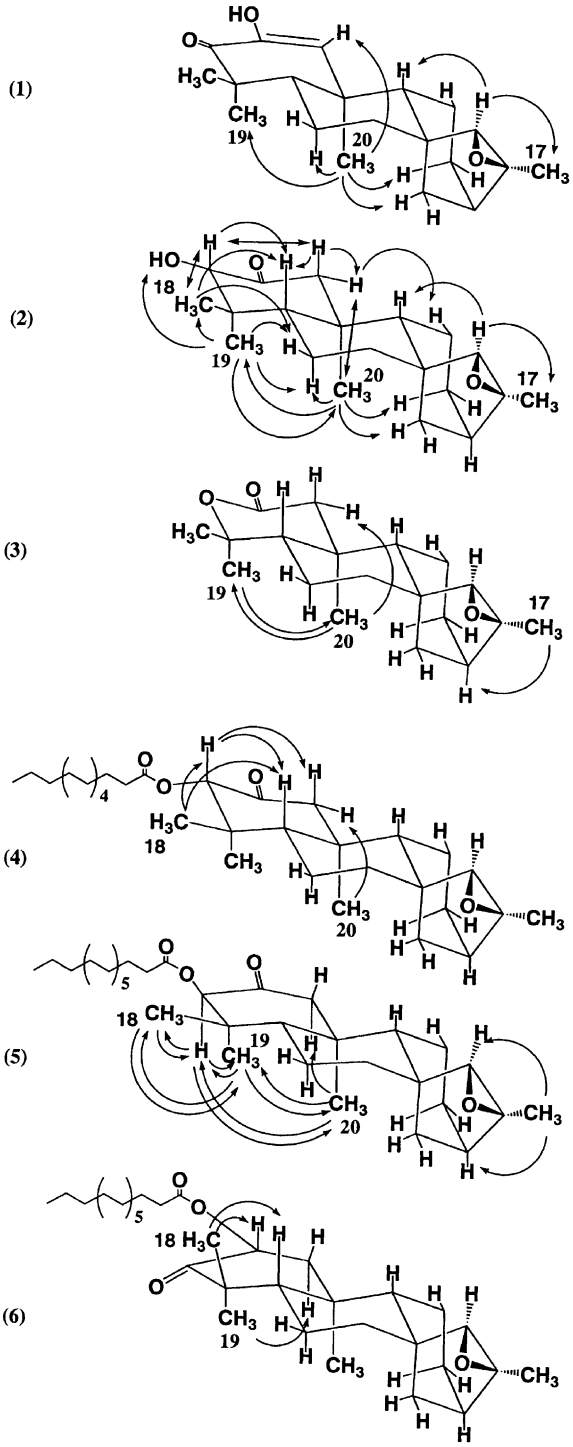


Fig. 3. Solution conformations of oryzadione (**1**), **2**, **3**, **4**, **5** and **6** were determined by NOEdiff and SELROESY(**2**).

2.3. Antibacterial activity of *1, 2, 3, 4 and 6*

Oryzadione (**1**), **2**, **3**, **4** and **6** inhibited approximately 40%, 30%, 45% 30% and 45% of colony formation of *X. campestris* pv. *oryzae* (Kono et al., 1991) at 200 ppm of **1** and **2**, 210 ppm of **3**, and 500 ppm of **4** and **6**, respectively.

3. Experimental

3.1. General experimental procedures

Standard analytical procedures used the following instruments: low- and high-resolution MS, Hitachi M-80; GC-SIM, JEOL-AUTOMASS150/Hewlett Packerd 5890 series II GC/Ultra Alloy capillary column (UA-1 HT; methyl silicone, 0.25 mm ID × 15 m × 0.25 μm film); ^{1}H and ^{13}C NMR spectra, JEOL GX-400 (400 and 100 MHz) and JEOL JNM-A600 (600 and 150 MHz); $[\alpha]_D$, Perkin–Elmer model 241 MC polarimeter; IR spectra, Shimadzu IR-435; UV spectra, Shimadzu UV-200; HPLC, Senshu SSC-3100 (Senshu Co., Ltd.; column: ODS-H-1151, 15 cm × 4.6 mm; detection: UV 270 nm). Thin-layer chromatography was carried out on Merck silica gel 60 F_{254} (0.25 or 0.5 mm in thickness; 20 cm × 20 cm). In prep. TLC (20 cm × 20 cm), 0.5 cm width of the TLC was cut by a glass cutter after being developed and the strip was colored with 5% vanillin-H_2SO_4 at 120 °C for 5 min.

3.2. Plants

Flag leaves (the uppermost leaf of the ripening stage) or leaves of the ripening stage of "Norin-27", a Japonica rice cultivar resistant against *X. campestris* pv. *oryzae* T7174, which was grown in a paddy field at Tokyo University of Agriculture and Technology in Fuchu-shi or was grown in concrete box (1/200 a), were collected as the source of the active substances.

3.3. Isolation procedures of *1*, *2* and *3*

Approximately 11 kg fresh weight of flag leaves was extracted with 80% aq MeOH at room temperature for one month after being cut into 3–5 cm pieces, and the MeOH extract solution was then collected by filtration and concentrated in vacuo to remove the MeOH. The concentrated aqueous solution (3 l) was adjusted to pH 8.4 with sodium carbonate and successively extracted with 2, 1 and 1 l of EtOAc, respectively. The combined EtOAc extract was concentrated to give 57 g of crude neutral substance. Half of this neutral residue was dissolved in EtOAc and silica gel (120 g) was poured into the solution. The slurry was concentrated in vacuo, and then the silica gel was layered on fresh silica gel (100 g) which was packed in a column (10 cm × 5 cm), that was successively eluted with 1 l of each of *n*-hexane, *n*-hexane–EtOAc (9:1), 100% $CHCl_3$ and $CHCl_3$–EtOAc (8:2), respectively. The eluate of $CHCl_3$–EtOAc (4.6 g) was injected into a silica gel column (120 g, 14 cm × 4.7 cm) and eluted with $CHCl_3$–EtOAc (95:5) to give 10 fractions which were collected according to the color reaction with 5% vanillin–H_2SO_4.

The third fraction (0.21 g) was applied to a Sephadex LH-20 column (70 cm × 3.5 cm) using MeOH as a solvent. An active fraction ($CHCl_3$–EtOAc 95:5, R_f 0.73) was collected (94.1 mg), and purified by prep. TLC (eluted with $CHCl_3$–EtOAc 95:5) using $CHCl_3$–EtOAc (95:5, R_f 0.73; yield 6.1 mg), *n*-hexane–EtOAc (7:3, R_f 0.69) and 100% $CHCl_3$ (R_f 0.31; 3.4 mg). An active compound was further purified using HPLC (gradient elution: 100% H_2O to 100% MeOH in 5 min, and 100% MeOH 25 min; 3 ml/min; R_t 10 min) to give 3.1 mg of **1** (approximately 0.6 mg/kg).

The fourth (0.16 g) and sixth fractions (0.85 g) were applied separately to the LH-20 column (70 cm × 3.5 cm) using MeOH as a solvent, and active compounds were collected, from each solution. An active fraction of the fourth fraction was purified by silica gel prep. TLC (eluted with $CHCl_3$–MeOH 9:1) using *n*-hexane–EtOAc (8:2, R_f 0.38), benzene–EtOAc (95:5, R_f 0.17) and *n*-hexane–EtOAc (8:2) to give 2.3 mg of pure compound **2**. An active fraction of the sixth fraction (151 mg) was purified by silica gel column (10 cm × 2 cm; $CHCl_3$–EtOAc, 85:15; 28 mg), and silica gel prep. TLC (eluted with $CHCl_3$–MeOH 9:1) using $CHCl_3$–MeOH (98:2: 7.2 mg), benzene–EtOAc (8:2, R_f 0.51; 3.9 mg) and benzene–EtOAc–MeOH (90:10:2, R_f 0.76) to give 0.9 mg of **2**. The total yield of **2** was 3.2 mg (0.58 mg/kg).

The fifth fraction (0.23 g) was purified by Sephadex LH-20 column (70 cm × 3.5 cm) using MeOH as a solvent and active fractions were collected. An active fraction (66.6 mg) was purified by silica gel prep. TLC (eluted with $CHCl_3$–MeOH 9:1) using benzene–EtOAc–MeOH (90:10:2, R_f 0.31; 22.5 mg), $CHCl_3$–EtOAc–MeOH (90:10:1, R_f 0.65; 7.2 mg), benzene–EtOAc (8:2; 3.7 mg) and *n*-hexane–EtOAc–MeOH (80:20:5, R_f 0.75) to give 1.2 mg of **3** (0.22 mg/kg).

3.4. Isolation procedures of *4*, *5* and *6*

Fresh leaves (1 kg) of ripening stage were extracted with 80% aq MeOH for one month after being cut into 3–5 cm pieces, and the MeOH extract solution was then collected by filtration and concentrated in vacuo to remove the MeOH. The water-soluble part of the concentrated solution was applied to an ODS column (25 cm × 5 cm). After the column was washed with 2 l of water, a water-insoluble solid material was dissolved in 20% MeOH and injected into the column, then the column was eluted with 2 l of 20% MeOH. The same procedure was repeated using 50% MeOH, 80% MeOH and 100% MeOH. In an experiment of quantitative determination of oryzalic acids A and B in rice leaves using GC-SIM, a precursor which provides oryzalic acid A by heat treatment (40 °C, 48 h), was observed in the 100% MeOH eluate of the ODS column (unpublished result). Therefore, GC-SIM and the color reaction with 5% vanillin–H_2SO_4 were used to detect the active compound.

The MeOH eluate was concentrated in vacuo to give 6.11 g of crude residue. The residue was dissolved in a minimum amount of MeOH and silica gel (20 g) was poured into the solution. The slurry was concentrated in vacuo, and then the silica gel was layered on fresh silica gel (20 g) which was packed in column. The column was eluted with 400 ml each of 100% *n*-hexane and then *n*-hexane–$CHCl_3$ (1:1).

The *n*-hexane–$CHCl_3$ (1:1) eluate was collected as the first and second eluates. The first eluate (0.69 g) was applied to a silica gel column (20 cm × 1.6 cm) using benzene–EtOAc (95:5) to give five fractions. The second of five fractions (26.5 mg) was further purified by prep.TLC (0.25 mm × 2) using *n*-hexane–benzene–MeOH (95:5:2) to give two fractions which contained R_f-low and R_f-high compounds. The R_f-low compound was purified by silica gel column (5 cm × 0.6 cm) using *n*-hexane–benzene–MeOH (80:20:2 and 80:20:5), *n*-hexane–EtOAc (9:1; R_f 0.14) and benzene–EtOAc (98:2; benzene–EtOAc 95:5, R_f 0.35) to give **4** (5.0 mg). The R_f-high compound was purified by silica gel column (5 cm × 0.6 cm) using benzene–EtOAc (9:1 and 98:2; R_f of **5/6** in 95:5: 0.48/0.40) and *n*-hexane–EtOAc (9:1, R_f of **5/6**: 0.21/0.25) to give **6** (5.0 mg) and an other compound. This second compound was further purified by silica gel column (5 cm × 0.6 cm) using *n*-hexane–EtOAc (9:1) to give 1.5 mg of **5**.

3.4.1. Oryzadione (*1*)

Colorless needle crystals. M.p. 138 °C; EIMS *m/z*: 316[M]$^+$; HREIMS Calculated for $C_{20}H_{28}O_3$: 316.2036; found: 316.2006; $[\alpha]_D^{24}$ −13° (c 0.21, MeOH); UV λ_{max} (MeOH) nm (ε): 273 (4800) and 205 (5000); IR ν_{max} (film) cm^{-1}: 3398(OH), 2925, 2850, 1664, 1648, 1443 (w), 1400, 1387, 1290 (w), 1247, 1050, 993, 835; ^{1}H (600 MHz, $CDCl_3$) and ^{13}C NMR (150 MHz, $CDCl_3$): Tables 1 and 2, respectively.

3.4.2. Compound *2*

Colorless needle crystals. M.p. 123 °C; EIMS *m/z*: 318[M]$^+$; HREIMS Calculated for $C_{20}H_{30}O_3$: 318.2211; found: 318.2202; $[\alpha]_D^{24}$ −5° (c 0.13, MeOH); UV λ_{max} (MeOH) nm (ε): 275 (185) and 215 (451); IR ν_{max} (film) cm^{-1}: 3530(OH), 2950, 2920, 2860, 1710 (C=O), 1440, 1388, 1170, 1140, 1110, 1100, 1053, 985, 945, 857, 838; ^{1}H (600 MHz, $CDCl_3$) and ^{13}C NMR (150 MHz, $CDCl_3$): Tables 1 and 2, respectively.

3.4.3. Compound *3*

Colorless needle crystals. M.p. 183 °C; EIMS *m/z*: 304[M]$^+$; HREIMS Calculated for $C_{19}H_{28}O_3$: 304.2036; found: 304.2021; $[\alpha]_D^{24}$ −54° (c 0.05, MeOH); UV λ_{max} (MeOH): end absorption; IR ν_{max} (film) cm^{-1}: 2940, 2920, 2870, 2850, 1708 (C=O), 1462, 1442, 13931367, 1322, 1300, 1110, 925, 840; ^{1}H (400 MHz, $CDCl_3$) and ^{13}C NMR (100 MHz, $CDCl_3$): Tables 1 and 2, respectively.

3.4.4. Compound *4*

Amorphous. EIMS *m/z*: 528[M]$^+$; HREIMS Calculated for $C_{34}H_{56}O_4$: 528.4175; found: 528.4147; $[\alpha]_D^{24}$ −33° (c 0.41, MeOH); IR ν_{max} (film) cm^{-1}: 2920, 2850, 1740 (O–C=O), 1730 (C=O), 1450, 1370, 1280, 1238, 1160, 1110, 1060, 985, 840; ^{1}H (600 MHz, $CDCl_3$) and ^{13}C NMR (150 MHz, $CDCl_3$): Tables 1 and 2, respectively.

3.4.5. Compound *5*

Amorphous. EIMS *m/z*: 556[M]$^+$; HREIMS Calculated for $C_{36}H_{60}O_4$: 556.4489 found: 556.4494; $[\alpha]_D^{24}$ −17° (c 0.14, MeOH); IR ν_{max} (film) cm^{-1}: 2920, 2850, 1740 (O–C=O), 1727 (C=O), 1460, 1375, 1280, 1238, 1150, 1110, 1060, 985, 840; ^{1}H (600 MHz, $CDCl_3$) and ^{13}C NMR (100 MHz, $CDCl_3$): Tables 1 and 2, respectively.

3.4.6. Compound *6*

Amorphous. EIMS *m/z*: 556[M] $^+$; HREIMS Calculated for $C_{36}H_{60}O_4$: 556.4489; found: 556.4496; $[\alpha]_D^{24}$ −67° (c 0.34, MeOH); IR ν_{max} (film) cm^{-1}: 2920, 2850, 1741 (O–C=O), 1728 (C=O), 1460, 1372, 1160, 1113, 1090, 982, 860, 840; ^{1}H (600 MHz, $CDCl_3$) and ^{13}C NMR (150 MHz, $CDCl_3$): Tables 1 and 2, respectively.

Acknowledgements

This work was supported in part by a Grant-in-Aid for Scientific Research (07459028) from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

References

Kono, Y., Uzawa, J., Kobayashi, K., Suzuki, Y., Uramoto, M., Sakurai, A., Watanabe, M., Teraoka, T., Hosokawa, D., Watanabe, M., Kondo, H.., 1991. Structures of oryzalides A and B, and oryzalic acid A, a group of novel antimicrobial diterpenes, isolated from healthy leaves of a bacterial leaf blight-resistant cultivar of rice plant. Agric. Biol. Chem. 55, 803–811.

Nakanishi, K., Watanabe, M., 1977a. Studies on the mechanisms of resistance of rice plants against *Xanthmonas oryzae*. III. Relationship between the rate of production of antibacterial substances and of multiplication of pathogenic bacteria in infected leaves of resistant and susceptible varieties. Ann. Phytopath. Soc. Jpn. 43, 265–269.

Nakanishi, K., Watanabe, M., 1977b. Studies on the mechanisms of resistance of rice plants against *Xanthmonas oryzae*. IV. Extraction and partial purification of antibacterial substances from infected leaves. Ann. Phytopath. Soc. Jpn. 43, 449–454.

Watanabe, M., Nakanishi, K., Kumakura, K., Shimoyama, N., Furukawa, M., 1976. Studies on the mechanisms of resistance in the rice plants infected with *Xanthomonas oryzae*. I. Inhibition of

lesion enlargement by the pre-inoculation with the non-pathogenic bacteria. Ann. Phytopath. Soc. Jpn. 42, 295–303.

Watanabe, M., Kono, Y., Esumi, Y., Teraoka, T., Hosokawa, D., Suzuki, Y., Sakurai, A., Watanabe, M., 1996. Studies on a quantitative analysis of oryzalides and oryzalic acids in rice plants by GC-SIM. Biosci. Biotech. Biochem. 60, 1460–1463.

Watanabe, M., Kono, Y., Watanabe, M., Uzawa, J., Teraoka, T., Hosokawa, D., Suzuki, Y., Sakurai, A., Teraguchi, M., 1992. Structures of oryzalic acid B and three related compounds, a group of novel antibacterial diterpenes, isolated from leaves of a bacterial leaf blight-resistant cultivar of rice. Biosci. Biotech. Biochem. 56, 113–117.

Watanabe, M., Sakai, Y., Teraoka, T., Abe, H., Kono, Y., Uzawa, J., Kobayashi, K., Suzuki, Y., Sakurai, A., 1990. Novel C_{19}-kaurane type of diterpene (oryzalide A), a new antimicrobial compound isolated from healthy leaves of a bacterial leaf blight-resistant cultivar of rice plant. Agric. Biol. Chem. 54, 1103–1105.