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ANTIFUNGAL ACTIVITY OF PISIFERIC ACID DERIVATIVES AGAINST THE RICE BLAST FUNGUS

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Key Word Index—*Chamaecyparis pisifera* var. *plumosa*; Cupressaceae; antifungal activity; structure–activity; computer graphic; pisiferic acid; probenazole.

Abstract—Twenty-eight derivatives of pisiferic acid (**1**), an antifungal constituent of *Chamaecyparis pisifera* var. *plumosa* against *Pyricularia oryzae*, were prepared and tested for activity. The size of the substituents at C-10 and C-12, as well as the presence and electronegativity of the constituent oxygen atoms, were inferred to be structural determinants for the activity of pisiferic acid derivatives. A computer graphic comparison between the derivatives and probenazole (**35**) (a commercial antifungal agent of the fungus) revealed the similarity of the size and location of the oxygen-containing substituents, which was supported by the similar mode of action of **1** and **35**.

INTRODUCTION

We have isolated the known diterpene carboxylic acid, pisiferic acid (**1**) and *O*-methylpisiferic acid (**2**) [1–5] from *Chamaecyparis pisifera* Endl. var. *plumosa* Beissn. (Cupressaceae) as antibiotics against Gram-negative bacteria, and found them to have antifungal activity against *Pyricularia oryzae* (rice blast fungus) (for **1**) and cytotoxicity against HeLa cells (for **1** and **2**) [6].

Our previous structure–activity relationship study [6, 7] indicated the C-10 carboxylic acid group (C-10 COOH) of **1** to be a primary structural factor for activity against *P. oryzae*. In order to induce a more precise structure–activity relationship, we prepared 28 derivatives (**3** and **7–33**) of **1** and tested them for antifungal activity against the fungus in the present study.

RESULTS AND DISCUSSION

General strategy for preparing the derivatives was directed to reduction of the C-10 COOH to CHO, CH_2OH and Me, and to derivitization of the C-12 OH to H, OMe and OCOR (mainly OAc). In order to clarify the effect of molecular size on activity, elongation was tried for COOR and CH_2OCOR' at C-10, and for OCOR at C-12 (for example, **5** → **8** → **10**, **17** → **25** → **29** and **12** → **14** → **15**).

The antifungal activity of the derivatives (**3** and **7–33**) and *m*-hydroxyphenylacetic acid (**34**) is listed in Table 1 together with that of the previously investigated compounds (**1**, **2** and **4–6**) [6, 7].

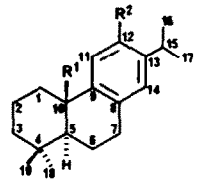
The following derivatives (expressed by pair of C-10 and C-12 substituents) showed strong activity (more than 65% inhibition): COOH–H (**40**) (not shown in Fig. 1) (65%) [7], COOH–OH (**1**) (85), COOMe–OH (**5**) (90), CHO–OH (**12**) (86), CHO–OAc (**14**) (79), CH_2OH–OH (**17**) (97), CH_2OH–OAc (**19**) (80) and CH_2OAc–H (**25**) (100); derivatives with CH_2OMe (**21–24**) and Me (**30–33**) at C-10 showed no or very weak activity independently of the C-12 substituents.

From the above, it is recognized that the electronegative oxygen atoms of COOH, COOMe, CHO and CH_2OAc are located in the C-10 substituents of the active derivatives as compared with the C-10 substituents (Me and CH_2OMe) of the inactive derivatives. For the C-10 CH_2OH, its hydrophobic property may affect activity besides its electronic property. Furthermore, molecular size depending upon the size of C-10 substituents affected activity, since activity decreased according to elongation of the substituent [**5** (90%) > **8** (37) > **10** (0)]. For C-12 substituents, OH enhanced activity when it was coupled with the C-10 substituents necessary for activity. On the other hand, the C-12 OAc induced high activity in the case of coupling with C-10 CHO (**14**) or CH_2OH (**19**). The other substituents [H (except for **25** and **40**) and OR] were unfavourable species for the C-12 substituents. The size of the substituents also influenced the activity [**12** (86% inhibition) > **14** (79) > **15** (8) and **17** (97) > **19** (80) > **20** (13)].

The C-10 COOH has been proposed to be a primary structural factor for antifungal activity because of the significant activity of **40** [7]. High activity of **25** suggests the C-10 CH_2OAc also to be an effective factor. However, since CH_2OAc–OH (**26**) and CH_2OAc–OAc (**28**) showed no activity, some other effects may be involved between COOH and CH_2OAc at C-10.

The inactivity of **26** (CH_2OAc–OH) and **16** (CH_2OH–H), and the high activity of **25** (CH_2OAc–H) and **17** (CH_2OH–OH) suggested the possibility that these activities were not due to hydrolysis of the C-12 CH_2OAc. Similarly, the extremely weak activity of **3** and **7** pointed out no hydrolysis reaction on the C-12 OAc because the corresponding C-12 OH compounds (**1** and **5**, respectively) showed strong activity.

For the inspection of stereostructure and molecular size, computer graphic analysis was carried out for the active derivatives (**1**, **5**, **12**, **14**, **17**, **19** and **25**) and an inactive one (**10**). Figure 2 (A) indicates the computer graphics for CPK-model of **1** viewed from frontal (*a*), top (*b*) and lateral (*c*) sides of the molecule. Figure 2 (B) shows



	R^1	R^2		R^1	R^2
1	COOH	OH	18	CH_2OH	OMe
2	COOH	OMe	19	CH_2OH	OCOMe
3	COOH	OCOMe	20	CH_2OH	$OCOC_2H_5$
4	COOMe	H	21	CH_2OMe	H
5	COOMe	OH	22	CH_2OMe	OH
6	COOMe	OMe	23	CH_2OMe	OMe
7	COOMe	OCOMe	24	CH_2OMe	OCOMe
8	$COOC_2H_5$	OH	25	CH_2OCOMe	H
9	$COOC_2H_5$	OC_2H_5	26	CH_2OCOMe	OH
10	$COOC_3H_7$	OH	27	CH_2OCOMe	OMe
11	CHO	H	28	CH_2OCOMe	OCOMe
12	CHO	OH	29	$CH_2OCOC_2H_5$	OH
13	CHO	OMe	30	Me	H
14	CHO	OCOMe	31	Me	OH
15	CHO	$OCOC_2H_5$	32	Me	OMe
16	CH_2OH	H	33	Me	OCOMe
17	CH_2OH	OH	34	m-Hydroxyacetic acid	

Fig. 1. Structures of pisiferic acid (1) and its derivatives (except 34).

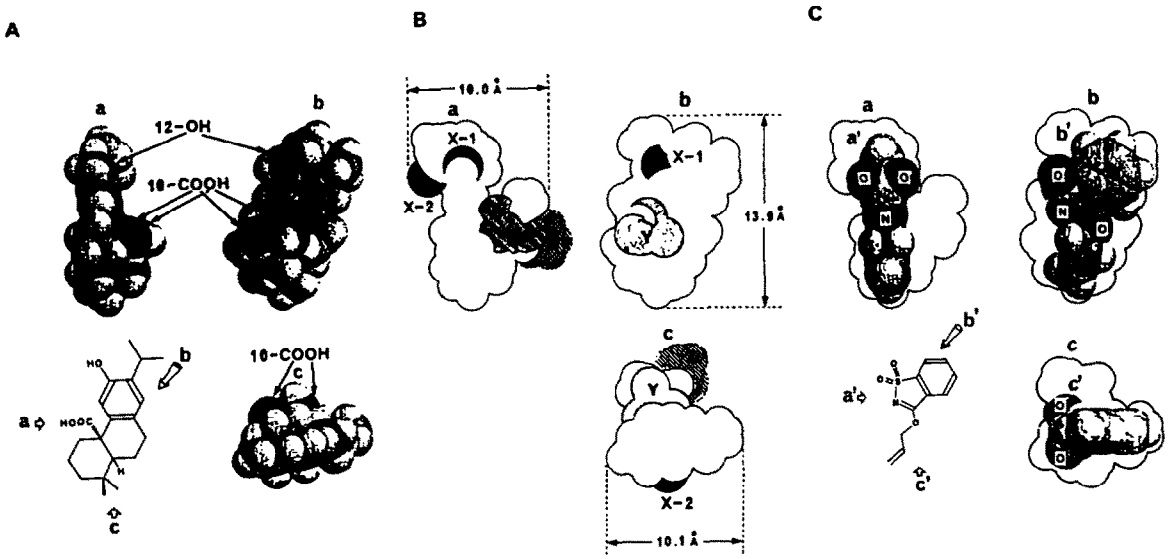
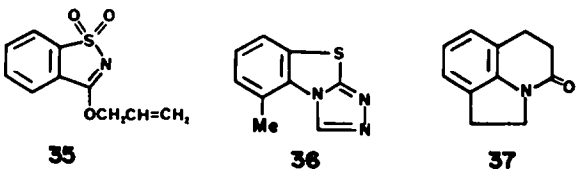


Fig. 2. CPK-model type computer graphics of pisiferic acid (1) (A) and probenazole (35) (C). (a), (b) and (c) in A represent the graphics viewed from frontal, top and lateral sides of the molecule of 1, respectively. (a'), (b') and (c') in C are similar viewpoints for 35. B shows the molecular framework of the active derivatives of 1 from the viewpoints (except Z-region). For X-1, X-2, Y and Z-region, see text.

the molecular profiles for the derivatives from each of the three aspects, these outlines accommodating all the active derivatives (except for the Z-region). However, in the case of the inactive **10**, the Z-region extends beyond this framework which suggests that **10** does not fit into the receptor space, accounting for its inactivity.

The electronegative oxygen atoms in the C-10 substituents are displayed in the Y-region in Fig. 2 (B). It was revealed that the O-atoms were located in a distinct area. A similar trend was observed for the O-atom of C-12 OH (X–1–region). However, the carbonyl O-atom (X-2) in the C-12 OAc of **14** and **19** was located in a different place



35 36 37

Fig. 3. Structures of the known antifungal agents against *P. oryzae*, which are probenazole (35), tricyclozole (36) and pyroquilon (37).

from the X-1, which implies the existence of different active sites in the receptor for the X–2 O-atoms.

Probenazole (35) [8], tricyclazole (36) [9] and pyroquilon (37) [10] are well known antifungal agents of *P. oryzae* and all possess a benzene ring in their structures as well as 1 (Fig. 3). Computer graphic inspection indicated similar molecular sizes for 35–37. Hence, 35 viewed from the three dimensions (a', b' and c') was compared with the active pisiferic acid derivatives for molecular size (Fig. 2C). 35 was readily accommodated within the space occupied by these derivatives (Fig. 2C, a–c). The X–1 and X–2 O-atoms were partially overlapped by either O-atom of the SO_2 of 35 in each viewpoint (Fig. 2C a, b and c, respectively), and the Y partially or completely to the N-atom of 35 (Fig. 2C a and b, respectively). 35 has been reported to be almost inactive (800 μg/ml) against *P. oryzae in vitro* [11], but considerably effective *in vivo* by inducing antifungal constituents from rice plants infected by the fungus [12]. Similarity in the molecular size and the location of the electronegative atoms in 35 and the antifungal pisiferic acid derivatives suggest an analogous mode of action between 35 and the derivatives. Therefore, the effect of 1 on the early infection process of *Pyricularia oryzae* to rice was examined with the slice-sheath inoculation method [13]. 35–37 were used as the reference test compounds.

In the presence of 1 (25 μg/ml), the intracellular hyphal growth of the fungus was remarkably suppressed with cytoplasmic denaturing of the infected cells. This aspect of the early infection stage was analogous to that of the infected cells treated with 35. In contrast, the fungus failed to penetrate into the epidermal cells with complete inhibition of appressorial pigmentation on treatment with 36 or 37. Details of this investigation will be reported elsewhere.

Considering a partial structure of 1 containing the C-10 COOH and phenol moieties, commercially available 34 was tested for activity; it exhibited some activity (Table 1). The other structural parts of 1 may, therefore, play a role for controlling the size and hydrophilicity–lipophilicity balance of the molecule to increase activity.

EXPERIMENTAL

Measurement of physical constants. Mps are uncorr. Optical rotations were measured at 25° in MeOH, unless otherwise stated. ^{1}H NMR were recorded in $CDCl_3$ with TMS (int std) at 90 MHz; all new compounds exhibited the expected spectra. MS were recorded at 70 eV.

Reactions. Chemical derivations were carried out by combination of the following reactions (a–h). Solvents used for reactions were dry. For purification of the reactants, prep. TLC was performed on precoated silica gel plates (60 F-254, Merck, 20 cm × 20 cm × 1 mm) eluting with *n*-hexane–Me_2CO mixts.

a. *Acylation.* A compound was treated with an equiv. vol. of Ac_2O or $(EtCO)_2O$ and pyridine for several hr at 0° or room temp.

Table 1. Antifungal activity of pisiferic acid (1) and its derivatives against *Pyricularia oryzae*

Compound	Antifungal activity (inhibition %)*	Compound	Antifungal activity (inhibition %)*
1	85†	18	31
2	0†	19	80
3	9	20	13
4	0‡	21	0
5	90†	22	6
6	0†	23	0
7	21	24	19
8	37	25	100
9	0	26	25
10	0	27	5
11	0	28	4
12	86	29	21
13	16	30	0
14	79	31	10
15	8	32	2
16	0	33	0
17	97	34§	25

*Average inhibition % spore germination at a dosage of 100 μg/ml of 3 tests, except for *m*-hydroxyphenylacetic acid (34).

†From ref. [6].

‡From ref. [7].

§Average inhibition % at 200 μg/ml of 3 tests.

b-1. *Alkylation*-1. To a soln of compound in hexamethylphosphoramide (HMPA), 25% NaOH was added and the mixt stirred for 1 hr at room temp. EtI or PrI was then added and stirring continued for 6 hr at room temp.

b-2. *Alkylation*-2. To a soln of NaH (50% mineral oil soln) in THF, MeI and compound in THF were successively added and stirred for 12–15 hr at 45–50°.

c. $LiAlH_4$ *reduction*. A compound and $LiAlH_4$ in Et_2O was stirred for *ca* 20 hr at room temp.

d. $NaBH_4$ *reduction*. A compound and $NaBH_4$ in EtOH was stirred for 1–1.5 hr at 0°.

e. *Modified Wolff–Kishner reduction*. A mixt. of compound, triethylene glycol (TEG), dry hydrazine [$(NH_2)_2$] and hydrazine dihydrochloride [$(NH_2)_2$·2HCl] was stirred for 14 hr at 140°. KOH was added at 0° and stirred for 7 hr at 150–220°. The mixt was poured into satd NaCl, extd with Et_2O and purified.

f. *Jones oxidation*. Jones reagent was added to a Me_2CO soln of compound at 0° and reacted for 3 min at room temp.

g. *Tetrahydropyranyl (THP) derivative* (**39**) ($R^1 = CH_2OH$, R^2 = OTHP) *from* **5**. Pyridinium *p*-toluenesulphonate (PPTS) (44 mg), dihydropyrane (199 mg) and **5** (180 mg) in CH_2Cl_2 (5 ml) was stirred for 6 hr at room temp. The mix was extd with Et_2O. The Et_2O soln (5 ml) was reacted with $LiAlH_4$ (83 mg) for 13 hr at room temp to give **39** which was used for subsequent steps without purification.

h. *Hydrolysis of OTHP group*. PPTS was added to a THP derivative in EtOH and stirred for 3 hr at 55°. Purification of the reactant gave **22**, **26** or **29**.

The following descriptions represent category of reactions (a–f or h), raw material, reaction conditions [reagent(s), solvent, temp, reaction period], eluting solving (hexane–Me_2CO) for prep. TLC, product and physical data.

Acetyl pisiferic acid (**3**). a, **1** (43 mg), Ac_2O/pyridine (0.2 ml each), room temp, 4 hr, 7:3, **3** (39 mg), amorphous; $[\alpha]_D$ + 138.5° (*c* 0.98); IR and ^1H NMR data were identical with those given in ref. [3].

Compounds **8** and **9**. b-1, **2** (160 mg), HMPA (1.25 ml), 25% NaOH (0.13 ml), EtI (0.27 ml), 9:1, **8** (67 mg) + **9** (114 mg). **8**, mp 95–96.5°; $[\alpha]_D$ + 131.0° (*c* 0.44); EIMS *m/z* (rel. int.): 344 $[M]^+$ (30), 271 $[M - CO_2Et]^+$ (100). **9** $[\alpha]_D$ + 122.4° (*c* 0.76); EIMS *m/z*: 372 $[M]^+$ (37), 299 $[M - CO_2Et]^+$ (100).

Compounds **10** *and* **38** ($R^1 = COOC_3H_7$, $R^2 = OC_3H_7$). b-1, **1** (160 mg), HMPA (1.25 ml), 25% NaOH (0.13 ml), PrI (0.2 ml), 9:1, **10** (77 mg) + **38** (111 mg). **10**, $[\alpha]_D$ + 134.7° (*c* 0.48); EIMS *m/z*: 358 $[M]^+$ (25), 271 $[M - CO_2C_3H_7]^+$ (100). **38**, $[\alpha]_D$ + 121.4° (*c* 0.87); EIMS *m/z*: 400 $[M]^+$ (34), 313 $[M - CO_2C_3H_7]^+$ (100).

Compound **7**. a, **5** (24 mg), Ac_2O/pyridine (0.1 ml each), 0°, 13 hr, 9:1, **7** (29 mg), $[\alpha]_D$ + 125.6° (*c* 1.25); IR ν_{max}^{neat} cm^{-1}: 1760, 1722; EIMS *m/z*: 372 $[M]^+$ (13), 271 (100).

Pisiferol (**17**). c, **5** (276 mg), $LiAlH_4$ (127 mg), Et_2O (14 ml), 7:3, **17** (258 mg), mp 114–115°; $[\alpha]_D$ + 64.7° (*c* 0.68); other data were identical with those given in ref. [14].

Pisiferal (**12**). f, **17** (35 mg), Jones reagent (2 drops), Me_2CO (1 ml), 85:15, **12** (22 mg), mp 135.5–136.5°; $[\alpha]_D$ + 265.2° (*c* 0.92), IR and ^1H NMR data identical with those in ref. [4].

Compound **14**. a, **12** (79 mg), Ac_2O–pyridine (0.5 ml each), 0°, 15 hr, 85:15, **14** (72 mg), $[\alpha]_D$ + 234.1° (*c* 1.15); IR ν_{max}^{neat} cm^{-1}: 1758, 1705; EIMS *m/z*: 342 $[M]^+$ (6), 313 $[M - CHO]^+$ (100).

Compound **19**. d, **14** (25 mg), $NaBH_4$ (2.8 mg), EtOH (1 ml), 4:1, **19** (23 mg), mp 85 – 87°; $[\alpha]_D$ + 60.7° (*c* 1.12); IR ν_{max}^{KBr} cm^{-1}: 3480, 1740; EIMS *m/z*: 344 $[M]^+$ (3), 271 (100).

Compound **15**. a, **12** (45 mg), $(EtCO)_2O$/pyridine (0.2 ml each), 0°, 14 hr, 9:1, **15** (45 mg), mp. 80.5 – 82°; $[\alpha]_D$ + 291.2° (*c* 0.63); EIMS *m/z*: 356 $[M]^+$ (8), 327 $[M - CHO]^+$ (100).

Compound **20**. d, **15** (24 mg), $NaBH_4$ (3 mg), EtOH (1 ml), 8:2, **20** (23 mg), mp 99 – 101°; $[\alpha]_D$ + 76.8° (*c* 0.69); EIMS *m/z*: 358 $[M]^+$ (1), 328 (100).

Ferruginol (**31**). e, **12** (140 mg), TEG (5 ml), $(NH_2)_2$ (2.4 ml), $(NH_2)_2$·2HCl (500 mg); KOH (2.6 g), 7 hr, 9:1, **31** (103 mg), amorphous; $[\alpha]_D$ + 64.0° (*c* 0.50); other data identical with those in ref. [1].

Ferruginol acetate (**33**). a, **31** (25 mg), Ac_2O/pyridine (0.2 ml each), 0°, 12 hr, 95:5, **33** (26 mg), $[\alpha]_D$ + 61.5° (*c* 0.78); IR and ^1H NMR identical with those in ref. [15].

Compound **28**. a, **17** (30 mg), Ac_2O/pyridine (0.1 ml each), room temp, 13 hr, 4:1, **28** (32 mg), $[\alpha]_D$ + 55.4° (*c* 0.33); IR ν_{max}^{neat} cm^{-1}: 1760, 1740; EIMS *m/z*: 386 $[M]^+$ (9), 271 (100).

Compound **22**. b-2, **39** (104 mg), NaH (39 mg), THF (8 ml), MeI (190 mg), 12 hr, 9:1, CH_2OMe–OTHP (69 mg); h, CH_2OMe–OTHP (69 mg), PPTS (4 mg), EtOH (2 ml), 9:1, **22** (48 mg), mp 150–152°; $[\alpha]_D$ + 62.7° (*c* 0.75); IR ν_{max}^{KBr} cm^{-1}: 3300, 1420; EIMS *m/z*: 316 $[M]^+$ (11), 271 $[M - CH_2OMe]^+$ (100).

Compound **24**. a, **22** (23 mg), Ac_2O/pyridine (0.2 ml each), 0°, 12 hr, 95:5, **24** (24 mg), $[\alpha]_D$ + 50.0° (*c* 0.84); IR ν_{max}^{neat} cm^{-1}: 1760; EIMS *m/z*: 358 $[M]^+$ (2), 313 $[M - CH_2OMe]^+$ (100).

Compound **26**. a, **39** (54 mg), Ac_2O/pyridine (0.2 ml each), room temp., 15 hr, crude CH_2OAc-OTHP (61 mg); h, CH_2OAc–OTHP (61 mg), PPTS (4 mg), EtOH (2 ml), 85:15, **26** (47 mg), amorphous: $[\alpha]_D$ + 35.9° (*c* 1.14); IR ν_{max}^{KBr} cm^{-1}: 3400, 1710; EIMS *m/z*: 344 $[M]^+$ (18), 271 $[M - CH_2OAc]^+$ (100).

Compound **29**. a, **39** (39 mg), $(EtCO)_2O$ (0.02 ml), pyridine (0.1 ml), room temp, 13 hr, crude CH_2OCOEt–OTHP; h, CH_2OCOEt–OTHP, PPTS (3 mg), EtOH (1 ml), 9:1, **29** (34 mg), $[\alpha]_D$ + 31.9° (*c* 1.44); EIMS *m/z*: 358 $[M]^+$ (65), 271 $[M - CH_2OCOEt]^+$ (100).

Compound **16**. c, **4** (386 mg), $LiAlH_4$ (186 mg), Et_2O (14 ml), 9:1, **16** (350 mg), $[\alpha]_D$ + 65.0° (*c* 1.55); IR ν_{max}^{neat} cm^{-1}: 3410; EIMS *m/z*: 286 $[M]^+$ (1), 255 $[M - CH_2OH]^+$ (100).

Compound **11**. f, **16** (195 mg), Jones reagent (appropriate), Me_2CO (4 ml), 97:3, **11** (153 mg), $[\alpha]_D$ + 350.0° (*c* 1.51); IR ν_{max}^{neat} cm^{-1}: 1710; EIMS *m/z*: 284 $[M]^+$ (1), 225 $[M - CHO]^+$ (100).

Compound **30**. e, **11** (76 mg), TEG (2.5 ml), $(NH_2)_2$ (1.2 ml), $(NH_2)_2$·2HCl (250 mg); KOH (1.3 g), 10:0, **30** (62 mg), $[\alpha]_D$ + 52.8° ($CHCl_3$; *c* 1.03); EIMS *m/z*: 270 $[M]^+$ (34), 255 $[M - Me]^+$ (100).

Compound **21**. b-2, **16** (41 mg), MeI (82 mg), NaH (14 mg), THF (4 ml), 15 hr, 95:5, **21** (37 mg), $[\alpha]_D$ + 64.8° ($CHCl_3$; *c* 1.23); IR ν_{max}^{neat} cm^{-1}: 1455, 1105; EIMS *m/z*: 300 $[M]^+$ (1), 255 $[M - CH_2OMe]^+$ (100).

Compound **25**. a, **16** (30 mg), Ac_2O/pyridine (0.5 ml each), room temp., 15 hr, 95:5, **25** (30 mg), $[\alpha]_D$ + 57.0° (*c* 1.48); IR ν_{max}^{neat} cm^{-1}: 1743; EIMS *m/z*: 328 $[M]^+$ (1), 255 $[M - CH_2OAc]^+$ (100).

Compound **18**. c, **6** (810 mg), $LiAlH_4$ (357 mg), Et_2O (30 ml), 8:2, **18** (682 mg), $[\alpha]_D$ + 69.3° (*c* 0.51); IR ν_{max}^{neat} cm^{-1}: 3440, 1460, 1250; EIMS *m/z*: 316 $[M]^+$ (21), 285 $[M - CH_2OH]^+$ (100).

Compound **13**. f, **18** (300 mg), Jones reagent (appropriate), Me_2CO (3 ml), 9:1, **13** (187 mg), $[\alpha]_D$ + 280.9° (*c* 0.47); IR ν_{max}^{neat} cm^{-1}: 1710, 1460, 1250; EIMS *m/z*: 314 $[M]^+$ (15), 285 $[M - CHO]^+$ (100).

Compound **32**. e, **13** (64 mg), TEG (2.5 ml), $(NH_2)_2$ (1.2 ml), $(NH_2)_2$·2HCl (250 mg); KOH (1.3 g), 95:5, **32** (48 mg), $[\alpha]_D$ + 68.5° (*c* 1.24); EIMS *m/z*: 300 $[M]^+$ (71), 285 $[M - Me]^+$ (100).

Compound **23**. b–2, **18** (50 mg), MeI (0.05 ml), THF (4 ml), 95:5, **23** (34 mg), mp 88.5–91.5°; $[\alpha]_D$ + 54.2° (*c* 1.13); EIMS *m/z*: 330 $[M]^+$ (17), 285 $[M - CH_2OMe]^+$ (100).

Compound **27**. a, **18** (19 mg), Ac_2O–pyridine (0.2 ml each), 0°,

14 hr, 95:5, 27 (20 mg), $[\alpha]_D$ + 45.7° (*c* 0.53); IR ν_{max}^{neat} cm^{-1}: 1740, 1460, 1230; EIMS *m/z*: 358 $[M]^+$ (18), 285 $[M - CH_2OAc]^+$ (100).

Compound **34** was obtained commercially.

Antifungal test. The method was that according to ref. [16]. *P. oryzae* F67–45 was cultured on an oat meal medium (Quaker Oats Co.) for 10–14 days at 25° in dark. On a welled-glass slide, the fungus (400–600 spores) in broth (20 μl) was mixed with a sample soln (50–400 μg of a compound in 20 μl of mixture of 2% EtOH and 3200 ppm Tween 80). The slide was incubated for 24 hr at 25° in the dark and then observed under the light microscope to determine the percentage of normal germination.

Computer graphics. The program 'MODRAST' made by Dr H. Nakano, Himeji Institute of Technology [17], was used for illustrating CPK type computer graphics (Fig. 3) of **1**, **5**, **8**, **10**, **12**, **14**, **15**, **17**, **19**, **20**, **25** and **35–37**.

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