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β-GLUCAN BIOSYNTHESIS INHIBITORS ISOLATED FROM FUNGI AS HYPHAL MALFORMATION INDUCER

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Abstract : Three hyphal malformation inducers were isolated from fungi and their structures identified as (+)-isoepoxydon (**1**), brefeldin A (**2**) and ophiobolin A (**4**), respectively. Two of them, **1** and **4**, were shown to be β-1,3-glucan synthetase inhibitors.

The chemical composition of cell walls is specific for each living thing, such as bacteria, fungi, or higher plants. Since mammalians like humans and livestock have no cell walls, they have been the intrinsic targets to develop highly selective and useful drugs in medicinal and agrochemical investigation. Penicillin thus marked a splendid milestone in the history of antibacterial drug development, that inhibited peptideglycan biosynthesis of bacterial cell wall. Polyoxin was found as a potent antifungal antibiotic by inhibiting selectively chitin biosynthesis of certain fungal cell wall[1]. Although insects have no cell wall but their body surface being covered with a typical cuticle layer, its selective inhibition became a prominent subject to produce a new type of synthetic insecticides characterized as an insect growth regulator. It is curious to note, however, that no practical herbicide has not been developed that inhibits cellulose (or hemicellulose) biosynthesis of plant cell walls.

The chemical components of fungal cell wall are various in each class of fungi[2], e.g., cellulose-glucan (Oomycetes), cellulose-chitin (Hyphochytridiomycetes), chitosan-chitin (Zygomycetes), chitin-glucan (Ascomycetes and Basidiomycetes) and mannan-glucan (Saccharomycetaceae). *Phytophthora* genus, belonging to Oomycetes is one of the most harmful phytopathogenic fungi causing serious damage to many crops. The hyphal cell wall of this genus is composed with major β-glucan and minor cellulose, and the former glucan has the main β-1,3-glucosidic matrix of D-glucose polymer blanched by β-1,6-glucosidic linkage[3].

The present study is to find new biosynthetic inhibitors of β-1,3 or β-1,6-glucan from fungal metabolites by using *Phytophthora capsici*, a pathogenic fungus on green pepper as a test organism. These β-glucan inhibitors are expected to be chemical tools, not only to determine the exact matrix structure of the β-glucan in the cell wall, but also to produce the man-made β-glucan fragments with a controlled molecular size linked with either glucosidic linkage we selected. We then considered that the biosynthetic inhibitor of cell wall might cause

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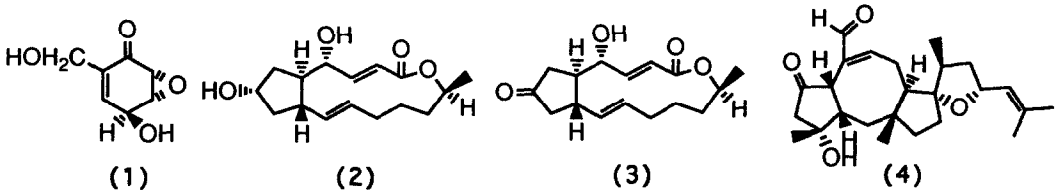
morphological malformation on growing hyphae, owing to a high inner pressure of the cell. Therefore, the first bioassay to be used for screening of many fungal cultures was a microscopic observation of hyphal malformation, and then once an active compound was isolated, its inhibitory activity on *in vitro* β-1,3-glucan synthetase was measured. We have examined more than 150 fungal strains that were cultured in the potato-dextrose agar medium. The aqueous acetone extract of the culture broth was adsorbed on a paper disc, and the disc was placed on the agar Petri dish 2 cm apart from the edge of the growing colony of *P. capsici*. Two days after the incubation, hyphal malformation was observed microscopically. Thus, three fungal strains, i.e. *Hemicarpenteles acanthosporus*, *H. paradoxus* and *Bipolaris sp.* were found to have induced unusual blanched and knotty hyphae around the paper disc. We then purified the active compounds produced in these fungal cultures.

The cultured broth of *H. acanthosporus* was extracted with ethyl acetate (EtOAc) and the EtOAc extract was purified twice by silica gel column chromatography with a stepwise elution of hexane (Hex)-EtOAc. The active compound (**1**) was isolated as an oil from the 60 % EtOAc fraction. This compound decomposed easily on standing at room temperature under air. The EI-MS spectrum did not show a molecular ion. ^{1}H- and ^{13}C-NMR spectra[4] showed the presence of each one of carbonyl and double bond, and oxygen bearing three methynes and one methylene. From these data, **1** was estimated to be (+)-isoepoxydon, being confirmed by its spectral coincidence with those of (+)-isoepoxydon reported[5,6].



(1) (2) (3) (4)

The cultured broth of *H. paradoxus* was extracted with EtOAc, and the EtOAc extract was purified by silica gel column chromatography with a stepwise elution of Hex-EtOAc. The active compound (**2**) was isolated as a crystal from the 60% EtOAc fraction. Another growth-inhibitory compound (**3**) was obtained from the 40% EtOAc fraction by further purifying with silica gel TLC and ODS HPLC. The spectral data[7] showed the presence of one ester carbonyl and each two of double bonds and oxygen bearing methylenes, suggesting **2** to be brefeldin A, whose spectral data in publication[8] coincided with those of **2**. Compound (**3**) showed the similar spectra in ^{1}H- and ^{13}C-NMR to brefeldin A (**2**), except the secondary alcohol carbon signal in the latter was replaced by the ketone (δ_C 215.7(s)) in **3**. Therefore, **3** should be 7-oxobrefeldin A, being ascertained by a coincidence of its spectral data with those of 7-oxobrefeldin A[9].

The cultured broth of *Bipolaris sp.* was extracted with EtOAc and the EtOAc extract was purified successively with silica gel column (EtOAc-Hex), silica gel TLC (EtOAc-CH_2Cl_2 (3:7)), and silica gel HPLC (EtOAc-CH_2Cl_2), affording an active compound (**4**) as a colorless crystal. The spectral data[10] indicate the presence of five methyls, each one of ketone, double bond and

α,β-unsaturated aldehyde, deducing **4** to be ophiobolin A, whose spectral data reported[11] were coincided with those of compound (**4**).

Now, (+)-isoepoxydon, brefeldin A and ophiobolin A have been identified as hyphal malformation inducers. Their minimum amounts to show activity were measured. As shown in Table (column I), ophiobolin A showed very strong activity, and the other two had half and one-tenth activity of ophiobolin A , respectively. Next, we examined their inhibitory activity of β-1,3-glucan synthetase *in vitro*. The crude enzyme of β-1,3-glucan synthetase was prepared by Cabib's method from *Saccharomyces cerevisiae* GS-1-36 which is the mutant lacking the ability of glycogen synthesis[12]. The crude enzyme, ^{14}C-UDP-glucose and a test sample were incubated together at 30℃ for 2 hr, and the uptake of radioactivity into the polymer fraction was measured. The results are shown in Table(column II); (+)-isoepoxydon and ophiobolin A showed the strong inhibition whereas brefeldin A did not.

Table The Biological Activities of (+)-Isoepoxydon, Brefeldin A and Ophiobolin A

	I[a]	II[b]						
	μg/disc	12.5	25	50	100	200	400	800 μg/ml
(+)-isoepoxydon	100	12	30	47	66	75	85	91 %
Brefeldin A	50	0	0	0	0	0	6	0 %
Ophiobolin A	10	31	56	56	74	79	84	89 %

a) Minimum amounts inducing hyphal malformation

b) Inhibition(%) of β-1,3-glucan synthetase from *S. cerevisiae*

That (+)-isoepoxydon was much weaker in hyphal malformation than ophiobolin A but similar to in the enzyme inhibition, might be ascribed to an easy degradation of (+)-isoepoxydon during the former *in vivo* bioassay that took two days, whereas the latter enzyme assay only 2 hrs. Brefeldin A, which was previously isolated as antibacterial[13] and antivirus antibiotic[14], was now found as a hyphal malformation inducer, but it did not inhibit β-1,3-glucan synthetase. Interestingly, 7-oxobrefeldin A with a ketonic group in place of the *sec*-OH in brefeldin A did not induce hyphal malformation but only hyphal growth inhibition was observed on *P. capsici*. (+)-Isoepoxydon was isolated as a germination inhibitor of lettuce seeds[6] and ophiobolin A as a growth inhibitor of higher plants[15], their phytotoxic activities might be correlated, at least in part, with the β-1,3-glucan synthetase inhibition, because a β-1,3-glucosyl linkage was shown to participate in an early stage of primary cell wall biosynthesis of higher plants[16].

In the present work, two of the three compounds that we have isolated as hyphal malformation inducers proved to be β-1,3-glucan synthetase inhibitors. This indicate that our first screening with a guidance of hyphal malformation could be fruitful to discover β--glucan synthetase inhibitors, although these two activities were not entirely in parallel. Our further efforts on this line produced recently another novel compound that was isolated from a fungus, being suggested to be the first β-1,6-glucan biosynthesis inhibitor found in nature.

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4. The physicochemical properties of **2** were as follows: $[\alpha]^{24}_{D}$ +190° (c 0.182, MeOH); UV λ_{max} (MeOH) nm(ε) : 212(5500), 239(3900); NMR δ_H (CD_3OD) : 3.46(1H, dd, J=1.3, 3.5 Hz), 3.78(1H, ddd, J=1.0,2.5, 3.5Hz), 4.25(2H ,AB type 15 Hz), 4.64(1H m), 6.70(1H, m); δ_C (CD_3OD) : 54.3(d), 58.9(d), 59.3(t), 63.4(d), 137.1(s), 139.7(s), 194.7(s).
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7. The physicochemical properties of **2** were follows : $C_{16}H_{24}O_4$(MS 280); mp 202.5℃; $[\alpha]^{24}_{D}$ +86.3° (c 1.53, MeOH); UV λ_{max} (MeOH) nm(ε) : 210(7500); MS(EI) m/z : 280(M^+), 262, 244; NMR δ_H($CDCl_3$) : 0.93(1H, m), 1.26(3H, d, J=6.3 Hz), 1.3-2.5(11H), 4.10(1H, m), 4.37(1H, m), 4.84(1H, m), 5.28(1H, dd, J=9.2, 15.1Hz), 5.70(1H, ddd, J=4.6, 10.0, 15.1Hz), 5.90(1H, dd,J= 1.9, 15.6Hz), 7.35(1H, dd, J=3.1, 15.6 Hz); δ_C (DMSO-d_6) : 20.7(q), 26.4(t), 31.5(t), 33.4(t), 40.9(t), 43.0(t), 43.3(d), 51.7(d), 70.4(d), 70.7(d), 74.2(d),116.1(d), 129.0(d), 136.9(d), 154.1(d), 165.4(s).
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10. The physicochemical properties of **4** were as follows: $C_{25}H_{36}O_4$ (MS 400); mp 182℃; $[\alpha]^{24}_{D}$ +280° (c 0.165, $CHCl_3$); UV λ_{max} (MeOH) nm(ε) : 236(10100); MS(EI) m/z : 400(M^+), 382, 353 ; NMR δ_H ($CDCl_3$) : 0.82(3H, s), 1.08(3H, d, J=7.0Hz), 1.36(3H, s), 1.70 (3H,s), 1.73(3H,s), 2.49 and 2.79(2H, AB type, J=19.2Hz), 3.26(1H, d, J=10.2Hz), 4.42(1H, m), 5.15(1H, brd., J=10.2Hz), 7.20(1H, t, J=8.4 Hz), 9.23(1H,s) ; δ_C($CDCl_3$) : 17.9, 18.2, 18.2, 23.6, 25.8, 25.9, 30.1,35.3, 36.8,40.9, 42.6, 43.1, 48.4, 50.2, 54.8, 60.5, 70.8, 76.6, 94.6, 125.2, 135.9, 141.5, 162.5, 195.5, 216.6.
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