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CYCLO-(L-TRYPTOPHYL-L-PHENYLALANYL), A PLANT GROWTH REGULATOR PRODUCED BY THE FUNGUS *PENICILLIUM* SP.

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Key Word Index—*Penicillium* sp.; fungus; cyclo-(L-tryptophyl-L-phenylalanyl); plant growth regulator.

Abstract—Cyclo-(L-tryptophyl-L-phenylalanyl) was isolated from an unidentified *Penicillium* sp. The absolute configuration of the compound was confirmed to be *S*, *S* at C-11 and C-14 by means of the comparison of the CD spectrum with that of the synthetic compound synthesized from L-tryptophan and L-phenylalanine. Cyclo-(L-try-L-Phe) and its components, L-tryptophan and L-phenylalanine, exhibited different biological activities against the tested plants of pine and tea pollen, lettuce, cress and rice seeds. Therefore, the biological activities of cyclo-(L-Trp-L-Phe) result from the intact compound and not the degradation to L-tryptophan and L-phenylalanine.

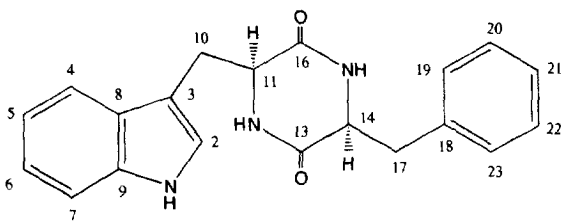
INTRODUCTION

We have investigated fungal metabolites for plant growth regulatory properties by bioassay methods using tea and pine pollen grains, and lettuce, cress and rice seeds, because such regulators may be suitable for developing new effective herbicides and for use as tools to analyse the growth of the organs in higher plants. Hence, we have elucidated the structures of the compounds, *O*-methyldihydrobotrydial [1], deacetyl-*O*-methyldihydrobotrydialone [1], rosellichalasin [2], pinthunamide [3], 7-hydroxy-2-hydroxymethyl-5-methyl-4H-chromen-4-one [4], altechromones A and B [5] and BSF-A [6] as growth regulators of lettuce seedlings. We have also elucidated the structures of the compounds, naphthoquinones [7], vulculic acid [8], hericerine [9] and emeniveol [10] as pollen growth and germination inhibitors. Furthermore, we have found a plant growth regulator in the culture filtrate of *Penicillium* sp., and isolated cyclo-(L-tryptophyl-L-phenylalany) (**1**) [11–14]. We have studied on the effects of cyclo-(L-Trp-L-Phe), L-tryptophan and L-phenylalanine against plant growth using tea and pine pollen grains, lettuce, cress and rice seeds.

RESULTS AND DISCUSSION

Isolation and the structure determination of cyclo-(L-tryptophyl-L-phenylalanyl)

Penicillium sp. was cultured stationarily in a potato extract medium containing sucrose at 24° for 21 days (see Experimental for details). The culture broth was filtered and the filtrate was adjusted to pH 2.0 with dilute HCl solution. Subsequently, the filtrate was extracted with ethyl acetate. After evaporating the solvent, the residue was dissolved with ethyl acetate and partitioned with saturated aqueous sodium bicarbonate solution. The organic layer was concentrated *in vacuo*, and the residue was chromatographed on a silica gel column. The active fraction eluted with hexane–acetone (35:65) was recrystallized from methanol give a powder, mp above 280°, in a yield of 12 mg.



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The molecular formula of the active compound was determined by HREI-mass specrometry ($[M]^+$ observed, *m/z* 333.1453, calculated, 333.1475) to be $C_{20}H_{19}N_3O_2$. This formula indicated 13 degrees of unsaturation. The infrared, ^{1}H NMR and ^{13}C NMR spectra of the compound showed several partial structures. The presence of two secondary amide groups in the molecule was suggested by an infrared band at 1669 cm^{-1} together with signals at δ 7.71 (1H, *d*) and 7.91 (1H, *d*) in the ^{1}H NMR spectrum and signals at δ 166.9 and 166.2 in the ^{13}C NMR spectrum. The presence of an indole ring was indicated by the charactistic fragmentation peak (*m/z*

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130) in the mass spectrum and by the positive reaction to the Ehlrich reagent. The mono-substituted phenyl ring was indicated by three aromatic hydrogens δ6.71 (2H, *m*), 7.16 (2H, *m*) and 7.17 (1H, *m*) and two infrared bands at 745 and 702 cm^{-1}. From the results of the measurement of the two-dimensional spectrum (H–H COSY), the methylene group (δ2.52 and 2.81) was linked to the methine group (δ3.98) adjacent to the amino group (δ7.91). In the same way, the methylene group (δ1.85 and 2.45) was linked to the methine group (δ3.89) adjacent to the amino group (δ7.71). On the basis of these partial structures and the other physicochemical data, the compound was identified as cyclo-(L-trptophyl-L-phenylalanyl) (1) [11–14].

In order to determine the absolute configuration of this compound, cyclo-(L-Trp-L-Phe) was synthesized from L-tryptophan and L-phenylalanine [15], and the comparison of the CD spectrum of the natural compound and the synthetic one confirmed the (*S*,*S*)-cofiguration at positions C-11 and C-14.

Biological activities of cyclo-(L-tryptophyl-L-phenylalanyl)

The biological activites of cyclo-(L-Trp-L-Phe) and its components, L-tryptophan and L-phenylalanine, were examined by the bioassay methods using pine and tea pollen, lettuce, cress and rice seeds.

Cyclo-(L-Trp-L-Phe) showed no inhibitory activity against pine pollen germination at a concentration of 100 $mg\,l^{-1}$ (Fig. 1), but L-tryptophan and L-phenylalanine showed weak inhibitory activity and weak accelerated activity against the pollen germination at the same concentration, respectively. Cyclo-(L-Trp-L-Phe) and L-phenylalanine showed no inhibitory activity against lettuce seed germination at a concentration of 100 $mg\,l^{-1}$, but L-tryptophan inhibited the germination by 62% at same concentration. Conversely, cyclo-(L-Trp-L-Phe) completely inhibited cress seed germination at a concentration of 100 $mg\,l^{-1}$ L-tryptophan inhibited the germination by 52% at the same concentration, but L-phenylalanine showed no inhibitory activity.

Cyclo-(L-Trp-L-Phe) inhibited tea pollen growth by 55% at a concentration of 100 $mg\,l^{-1}$ (Fig. 2). However, L-tryptophan and L-phenylalanine showed no inhibitory activity at the same concentration. Three compounds showed no inhibitory activity against the hypocotyl elongation of lettuce seedlings at a concentration of 100 $mg\,l^{-1}$ (Fig. 3). Cyclo-(L-Trp-L-Phe) accelerated the root growth of the seedlings in proportion to concentration from 1 $mg\,l^{-1}$ to 100 $mg\,l^{-1}$, but L-tryptophan and L-phenylalanine showed no inhibitory activity. The three compounds showed no inhibitory activity against the hypocotyl elongation of cress seedlings at a concentration of 100 $mg\,l^{-1}$ (Fig. 4). They showed weak inhibitory activity against the root growth of the seedlings at a concentration of 100 $mg\,l^{-1}$. The three compounds showed little inhibitory activity against the second leaf sheath elongation and the root growth of rice seedlings at a concentration of 100 $mg\,l^{-1}$ (Fig. 5).

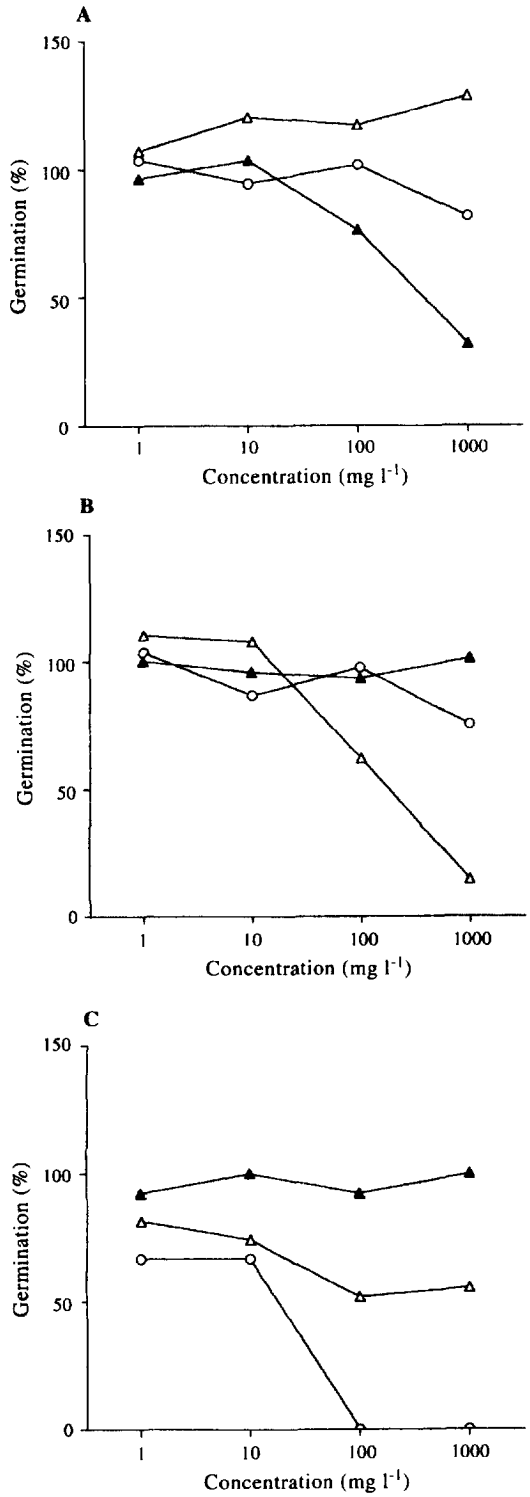


Fig. 1. Effects of cyclo-(L-Trp-L-Phe) (○), L-Try (△) and L-Phe ▲ on the germination of pine pollen (A), lettuce (B) and cress seeds (C).

From the results described, cyclo-(L-Trp-L-Phe) and its components, L-tryptophan and L-phenylalanine, exhibited different biological activities against the tested plants. Therefore, the biological activities of cyclo-(L-

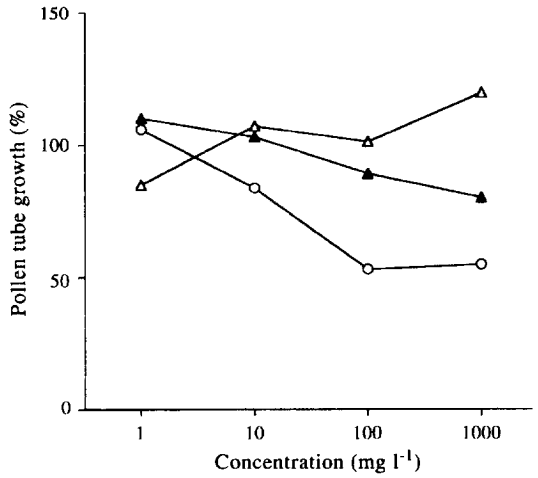
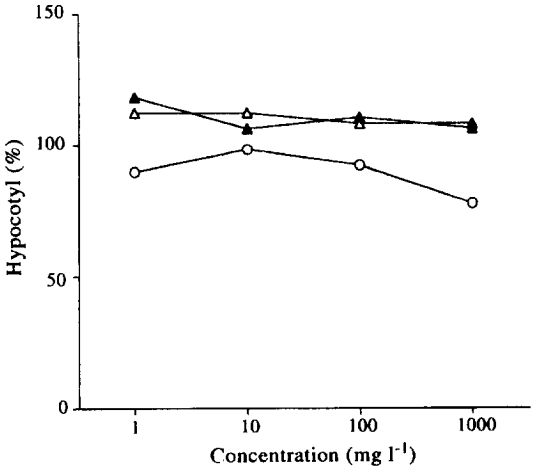


Fig. 2. Effects of cyclo-(L-Trp-L-Phe) (○), L-Try (△) and L-Phe ▲ on the growth of tea pollen.



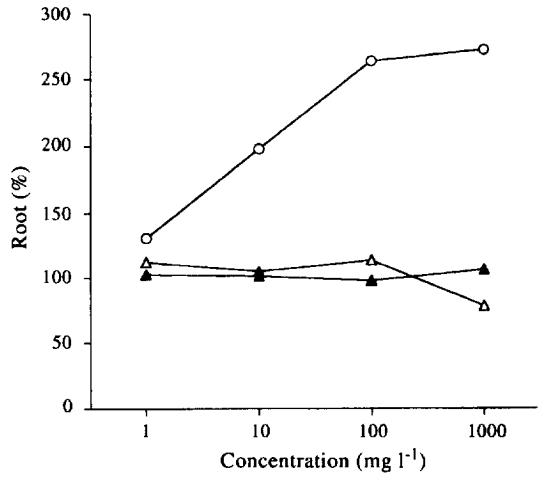
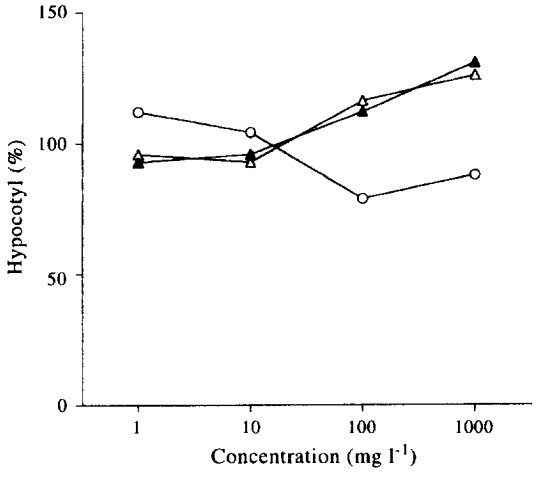


Fig. 3. Effects of cyclo-(L-Trp-L-Phe) (○), L-Try (△) and L-Phe ▲ on the growth of lettuce seedlings.

Trp-L-Phe) could not result from degradation to L-tryptophan and L-phenylalanine but are properties of the intact dipeptide.



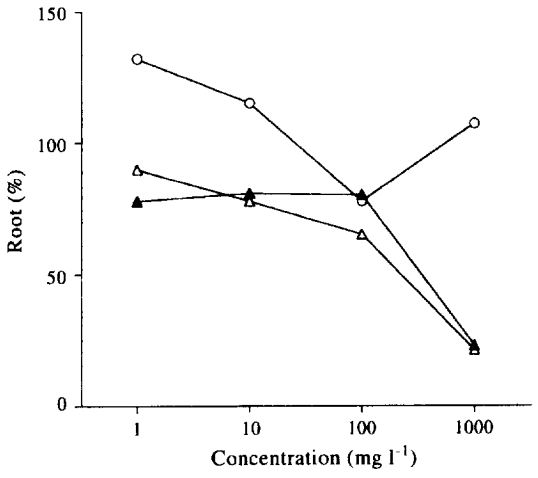


Fig. 4. Effects of cyclo-(L-Trp-L-Phe) (○), L-Try (△) and L-Phe ▲ on the growth of cress seedlings.

EXPERIMENTAL

General. The IR spectrum was recorded as a KBr pellet. The H NMR spectrum was recorded at 270.05 MHz and the ^{13}C NMR spectrum at 67.80 MHz in DMSO-d_6 with TMS as int. standard. HRMS was recorded in the EI mode. CC was performed with silica gel (Wakogel C-200).

Extraction and isolation. One hundred and sixty Erlenmeyer flasks (500 ml), each flask containing 250 ml of a potato sucrose medium, made from potato (200 g l^{-1}) and sucrose (30 mg l^{-1}) were inoculated with spores of *Penicillium* sp. previously grown on solid potato sucrose agar. The culture broth (40 l) was grown stationarily at 24° for 21 days and then filtered to separate the mycelium from the broth. The filtrate was adjusted to pH 2.0 with dil. HCl soln. Subsequently, the filtrate was successively extracted with EtOAc. After evaporating the solvent, the residue (10.7 g) was dissolved with EtOAc and partitioned with aq. satd $NaHCO_3$ soln. The organic layer was concentrated *in vacuo*, and the residue (3.4 g) was chromatographed on a silica gel column (hexane–Me_2CO). The active fraction (43.1 mg), eluting with

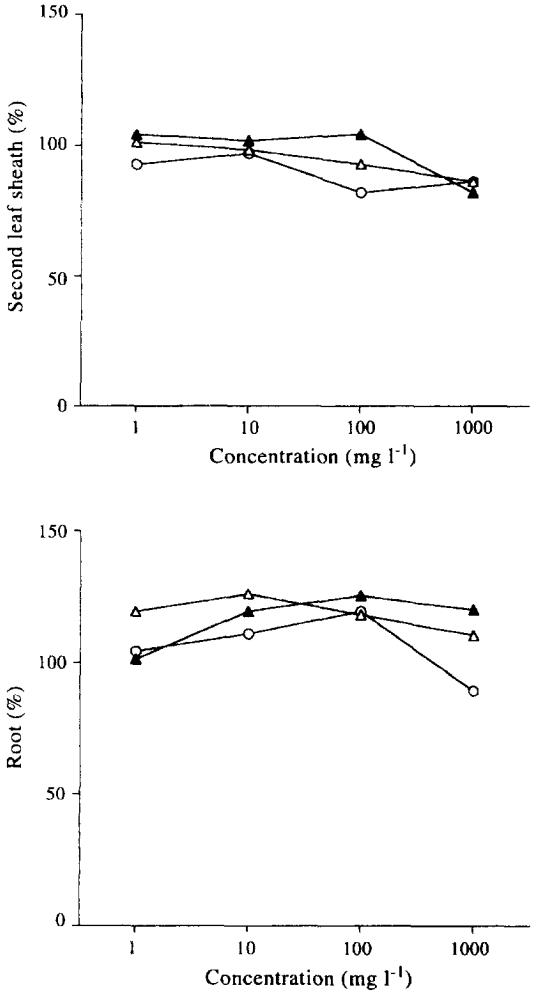


Fig. 5. Effects of cyclo-(L-Trp-L-Phe) (○), L-Try (△) and L-Phe ▲ on the growth of rice seedlings.

hexane–Me_2CO (35:65), was evapd to dryness and gave plates of cyclo-(L-Trp-L-Phe) (12 mg) after recrystallizing from MeOH.

Cyclo-(L-Trp-L-Phe). Powder, mp >280°. $[\alpha]_D^{20}$ − 254.9° (MeOH; *c* 1.0). CD: $[\theta]_{231}$ + 3363, $[\theta]_{244}$ − 1644, $[\theta]_{282}$ − 2046, $[\theta]_{290}$ − 2604 (MeOH; *c* 0.094). UV λ_{max}^{EtOH} nm: 289, 281, 272, 218, IR ν_{max}^{KBr} cm^{-1}: 3226, 3192, 3050, 1669, 1520, 1460, 1325, 1238, 745, 702. ^1H NMR (270.05 MHz, DMSO-d_6): δ1.85 (1H, *dd*, *J* = 13.49, 7.02 Hz, H-17), 2.45 (1H, *dd*, *J* = 13.49, 4.70 Hz, H-17), 2.52 (1H, *dd*, *J* = 14.47, 5.68 Hz, H-10), 2.81 (1H, *dd*, *J* = 14.47, 4.46 Hz, H-10), 3.89 (1H, *m*, H-14), 3.98 (1H, *m*, H-11), 6.71 (2H, *m*, H-20, 22), 6.96 (1H, *d*, *J* = 2.20 Hz, H-2), 6.98 (1H, *ddd*, *J* = 7.17, 7.45, 1.45 Hz, H-5), 7.08 (1H, *ddd*, *J* = 7.73, 7.45, 1.00 Hz, H-6), 7.16 (2H, *m*, H-19, 23), 7.17 (1H, *m*, H-21), 7.32 (1H, *dd*, *J* = 7.73, 7.45 Hz, H-7), 7.48 (1H, *dd*, *J* = 7.17, 1.00 Hz, H-4), 7.71 (1H, *d*, *J* = 2.00 Hz, H-15), 7.91 (1H, *d*, *J* = 2.00 Hz, H-12), 10.89 (1H, *s*, H-1). ^{13}C NMR (67.80 MHz, DMSO-d_6): δ29.69 (*t*, C-10), 39.89 (*t*, C-17), 55.29 (*d*, C-11), 55.64 (*d*, C-14), 108.85 (*s*, C-3), 111.33 (*d*, C-7), 118.42 (*d*, C-4), 118.76 (*d*, C-5), 120.89 (*d*, C-2), 124.41 (*d*, C-6), 126.36 (*d*, C-21), 127.54 (*s*, C-8), 128.03 (*d*, C-19, 23), 129.70 (*d*, C-20, 22), 136.07 (*s*, C-9), 136.56 (*s*, C-18), 166.22 (*s*, C-13), 166.88 (*s*, C-16). EIMS *m/z* (rel. int.): 333 $[M]^+$ (69), 240 $[M - 93]^+$ (8), 183 $[M - 150]^+$ (17), 130 $[M - 203]^+$ (100), 113 $[M - 220]^+$ (11). HREIMS ($[M]^+$ obs., *m/z* 333.1453, calcd. for $C_{20}H_{19}N_3O_2$, 333.1475).

Synthesis of cyclo-(L-tryptophyl-L-phenylalanyl). L-Tryptophan methyl ester hydrochloride and *N*(-*t*-butoxycarbonyl)-L-phenylalanine were allowed to react according to established procedures [15] to give L-tryptophyl-*N*-(*N*-(*t*-butoxycarbonyl)-L-phenylalanyl) methyl ester. The compound was allowed to react according to the procedures to give cyclo-(L-tryptophyl-L-phenylalanyl), mp > 280°. $[\alpha]_D^{20}$ − 254.9° (MtOH; *c* 1.0). IR ν_{max}^{KBr} cm^{-1}: 3422, 3188, 3054, 1669, 1460, 745, 702. EIMS *m/z* (rel. int.): 333 $[M]^+$.

Bioassay for the germination of pine pollen, lettuce and cress seeds. Pollen germination activities of the compounds were measured by the established method [7] using pollen grains of *Pinus thunbergii* Parl. The pollen germination after treatment with compounds was measured under a microscope and compared with the values for an untreated control.

The germination of lettuce seeds treated with compounds was measured by the established method [16]. Lettuce seed germination was measured 48 hr after treatment and compared with the values for an untreated control.

The germination of cress seeds treated with compounds was measured by the same method used for lettuce seeds. The cress seed germination was measured 40 hr after treatment and compared with the values for an untreated control.

Bioassay for the growth of tea pollen, lettuce, cress and rice seedlings. Pollen growth activities of the compounds were investigated by the method [17] using tea pollen grains of *Camellia sinensis* O. Kuntze. The length of tea pollen tubes after treatment with compounds was measured and compared with the values for an untreated control.

The growth of lettuce seedlings treated and the compounds was examined by method [16]. The lengths of the hypocotyls and root of the seedlings treated with the compunds were measured and compared with the values for an untreated control.

The growth of cress seedlings treated with the compounds was tested by the same method as that used with lettuce seedlings. The lengths of the hypocotyls and roots after treatment with the compounds were measured and compared with the values for an untreated control.

The growth of rice seedlings treated with the compounds was measured by the established method [18]. The lengths of the second leaf sheaths and roots after treatment with the compounds were measured and compared with the values for an untreated control.

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