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Isolation and Identification of Maculosins from *Streptomyces rochei* 87051-3

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Abstract : Herbicidal compounds were isolated from the fermentation broth of *Streptomyces rochei* 87051-3, and identified as maculosin-1, -5, and -6. This is the first report on a *Streptomyces* species that produces maculosins (Received September 30, 1994; accepted October 17, 1994)

Introduction

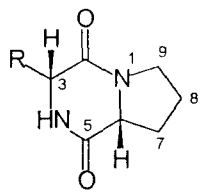
Use of microorganisms for the control of weeds in crop productions has been gaining acceptance in the agricultural practices as the environmental concerns in the agricultural products become acute. It is noteworthy that at least one microbial phytotoxin has been successfully developed into a commercial herbicide as seen with bialaphos.[1] Recent progress in this field has been well reviewed and documented elsewhere.[2] Maculosins, a family of at least seven related diketopiperazine compounds formally derived from condensation of proline and other amino acids, are known to be produced by the fungi, such as *Alternaria alternata*,[3] *Beauveria bassiana*[4] and *Fusarium nivale*.[5]

Recently, Kim *et al.* described a phytotoxic microorganism isolated from soil and identified it as *Streptomyces rochei*.[6] It was shown to produce phenylacetic acid.[7] This paper describes the identification of maculosins other than the acid as phytotoxic principles from *S. rochei*.

Materials and Methods

Strain

Strain used in the experiment was *S. rochei*



Maculosin -1, R=p-hydroxybenzyl
-2, R=benzyl
-3, R=benzyl, 3*S*
-4, R=isopentyl
-5, R=isopropyl
-6, R=isobutyl
-7, R=methyl

87015-3,[6] which was deposited in the culture collection at the Agricultural Chemicals Research Institute(Rural Development Administration, Suwon 441-707, Republic of Korea). Spores of the strain were harvested from cultures grown on yeast extract agar medium[8] and preserved in 50% glycerol at −20℃.

Reagents and solvents

All reagents used for the culture media were GR grade. Most solvents used in the extraction and chromatography were GR or redistilled EP grade. The adsorbent used for the vacuum liquid chromatography (VLC) was Silica gel 60 G (Merck, Germany).

Fermentation

An aliquot (3 m*l*) of spore suspension was inoculated into the 500 m*l* Erlenmeyer flasks containing 100 m*l* of medium A[7] and incubated at 27~30℃ on a shaking rotary incubator (150 rpm). For a large

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scale fermentation, approximately 200 ml of the culture was seeded into 10 l of the medium in a fermentor (Biostat E, B. Braun, Germany). The fermentation was carried out at 27℃, 200 rpm for three days.

Extraction and separation

Mycelia from culture broth (20 l) were removed through suction filtration. The filtrate was concentrated to approximately 1 l with a rotary vacuum evaporator. The concentrate was then extracted three times with EtOAc. Subsequently, the extract was sequentially extracted with 0.1 N HCl and 0.05 N NaOH. The remaining organic layer was dried under reduced pressure.

Vacuum liquid chromatography of the residue (180 mg) was performed with increasing stepwise gradient of ethanol in dichloromethane.[9] The dimension of VLC column used was 50×150 mm. The fractions were examined through TLC (EtOH-CH_2Cl_2, 1 : 3), the separated compounds on TLC plate being visualized by UV light and I_2 vapor.

Bioassay

Germination rate and stem length of raddish (cv. Chungsu, Nongwoo Seedling Co.) and rice (cv. Dongjin) seedlings were examined. A calculated amount of sample dissolved in ethanol solution was blotted on a filter paper in a Petri dish (diameter, 50 mm). After ethanol was evaporated by hot air stream, 2 ml of distilled water was added. Subsequently, 15 seeds were evenly placed on the paper and were incubated at 27℃ with light and darkness alternating at intervals of 16~8 hours. After 72 hours, germination rate and growth of stem were measured. All the bioassay experiments were done in triplicate.

Instruments

NMR spectra were determined on a Bruker AM-200 operating at 200 MHz for ^1H and 50 MHz for ^{13}C. Electron impact mass spectra(EI-MS) were obtained from VG 70-SEQ mass spectrometer equipped with DB-1-CC-24m column in either direct probe or GC/MS mode, and IR spectra were obtained with a Perkin-Elmer 1710 FT-IR instrument.

Results and Discussion

Maculosin-1.

PMR ($CDCl_3$) δ 7.04 (2H, d, J=8.3), 6.78 (2H, d, J=8.3), 6.00 (1H, s), 4.23 (1H, dd, J=9.5 and 3.5), 4.12 (1H, t, J=7.6), 3.55-3.61 (2H, m), 3.44 (1H, dd, J=3.5, 14.5), 2.78 (1H, dd, J=9.5 and 14.5), 2.31-2.35 (1H, m), 1.85-2.05 (3H, m); ^{13}C-NMR (acetone-d_6) δ 206.0 170.2, 157.5, 131.8, 116.3, 59.8, 57.4, 45.8, 36.5, 29.2, 23.2; IR(KBr) cm^{-1} 3380(br, s), 2956(m), 2880(m), 1661(s), 1507(s), 1446(m); EI-MS(70eV) m/z (rel. int.) 260(31, M^+), 204(7), 170(9), 154(100), 144(15), 125(15), 120(31), 113(11), 107(85), 91(32), 77(15), 70(55).

Maculosine-5.

PMR ($CDCl_3$) δ 6.00 (1H, s), 5.84 (1H, br. s), 4.01 (1H, t, J=9.0), 3.87 (1H, br. s), 3.4-3,65 (2H, m), 2.5-2.65 (1H, m), 2.25-2.4 (1H, m), 1.8-2.1 (3H, m), 0.99 (3H, d, J=7.2), 0.85 (3H, d, J=6.8); ^{13}C-NMR (acetone-d_6) δ 206.1, 173.4, 59.8, 45.8, 29.4, 29.3, 23.3, 19.2, 17.2; IR(KBr) cm^{-1} 3215(w), 2984(m), 2878(m), 1668(s), 1432(m), 1078(m), 913(w); EI-MS(70 eV) m/z (rel. int.) 196(12, M^+), 154(100), 138(12), 125 (78), 110(17), 98(20), 72(74).

Maculosine-6.

Not isolated, EI-MS (70 eV) m/z (rel. int.) 154 (100), 125(10), 86(18), 70(40).

The final EtOAc extract exihibited weak bioactivity. About 25 mg of crystal(needle) was obtained from VLC fractions 4 (5% ethanol) and 5 (7% ethanol) through recrystalization in cyclohexane. These fractions had 5~10% inhibition on raddish seed germination and 7~17% of inhibition on the stem growth. TLC done on the 30 mg of yellow oil, obtained from the fractions 9 (20% ethanol) and 10 (25% ethanol), showed the oil to be pure. These fractions exhibited inhibition of 12% on raddish seed germination and 23% on the stem growth.

The NMR spectroscopic examination of the oil

showed four phenyl protons at 6.78 (2H, d, J=8.3) and 7.04 (2H, d, J=8.3) with *para*-substitution pattern. A singlet at 6.00 ppm was due to a lactam group. Mass spectrum showed a base peak at m/z 154 due to a bicyclic ring moiety plus a hydrogen, a molecular ion at m/z 260, and 4-hydroxybenzyl moiety at m/z 107. Number of carbon atoms, the presence of a phenyl group and two carbonyl groups were confirmed with ^{13}C-NMR spectrum. Occurence of a hydroxy and an amide group was indicated by infrared absorptions at 3380 and 1661 cm^{-1}, respectively. The spectral data were consistent with the values of maculosin-1 as reported by Strobel *et al.*[3]

The ^{1}H-NMR spectrum of the oil and the crystal shared common signals, thus implying common feature in the structures. In the case of the crystal, two doublet signals of typical diastereotopic dimethyl group were observed at 0.99 and 0.85 ppm instead of signals of 4-hydroxy benzyl group in ^{1}H-NMR spectrum. Mass spectrum of this crystal showed the molecular ion at m/z 196 and a base peak at m/z 154 as with maculosin-1. An additional peak at 125, rather abundant, is believed to arise from a five-membered ring-containing fragment due to fission of C2-C3 and N4-C5. Inspection of the ^{13}C-NMR spectrum and IR spectrum gave the structure of the crystaline compound as maculosin-2. The spectral data were again consistent with the published ones.[3]

However, the peak at m/z 86 (abundance, 13%) observed in mass spectrum of the crystal cannot be interpreted from the structure of maculosin-5. When the crystal was analyzed by GC/MS, two peaks were found in TIC profile, roughly in 8 : 2 ratio. The ealier eluted compound had a peak at m/z 72 while the trailing compound at m/z 86. The fragment at m/z 72 or 86 was believed to originate from 1-aminoisobutyl moiety or 1-aminoisopentyl group which had arizen from fission of C2-C3 and N4-C5 of maculosin-5 and -6. Careful examination of the dimethyl regions in ^{1}H-NMR spectrum of the crystal also revealed contaminating peaks due to maculosin-6 (about 5%). The presence of the contaminating maculosin-6 was thus apparent. Since peaks cannot be directly compared quantitatively in a TIC, 5 percent of contamination, not 20 percent as estimated from TIC profile, due to maculosin-6 would be a more resonable estimation.

It is known that stereoisomers at C3 is possible as seen with maclosins-2 and -3. The optical rotation and hrms data of the compounds could be needed for further confirmation of the stereochemistry at C3 and identification. However, exact match of the NMR data with the previous report of Strobel's group[3,10] precluded the assignment of the structure other than the ones presented here, since such a stereoisomerism would induce discrepancies in the proton chemical shift values.

There has been three reports on the identification of maculosins from microbes; Tamura *et al.*[4] from *B. bassiana*, Tatsuno *et al.*[5] from culture of *F. nivale* and Stierle *et al.*[3] from *A. alternata*. Authencity of isolation reported by Tamura *et al.* and Tatsuno *et al.* was critically revoked by Strobel, who found that only maculosin-1 and -2 exhibited a host-specific phytotoxicity on spotted knapweed (*Centaurea maculosa*), which is major weeds in the northwestern United States, at a concentration as low as 10^{-5}M. Such a phytotoxicity of maculosin-1 was partly identified in this experiment where weak inhibition on the germination and stem growth of raddish seeds were observed at a concentration of 200 ppm. The observed phytotoxicity, albeit low toward the plants used in this study, means that maculosin(s) is not species-specific at rather higher concentration at about 10^{-3}M.

The present report of occurence of maculosins in the fermentation broth of *Streptomyces*, other than a fungus *Alternaria*, not counting *Beauveria* and *Fusarium*, is the first report of maculosins produced by an bacterium. We have reported that *S. rochei* 87051-3 also produces phenylacetic acid as another phytotoxic principle in the fementation broth.[7] It is thus now conceivable that phytotoxicity of the *Streptomyces* strain is due to the combined or synergistic effect[10] of maculosins and phenylacetic acid. Occurence of weak activity in the fractions

of the fermentation broth other than maculosins and phenylacetic acid was indicated.[7] Further study would be necessary to confirm the occurence of other maculosins and phytotoxins in *S. rochei* 870 51-3.

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Streptomyces rochei 87051-3에서 Maculosin류의 분리와 동정

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초록 : *Streptomyces rochei* 87051-3의 발효액에서 제초성 물질을 분리하고 maculosin-1, -5 및 -6으로 동정하였다. 이것은 *Streptomyces*에서 이들 물질이 분리된 최초의 보고이다.

찾는말 : *Streptomyces rochei*, maculosins, phytotoxicity