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Isolation of indole-3-acetic acid methyl ester, a metabolite of indole-3-acetic acid from *Pseudomonas amygdali*

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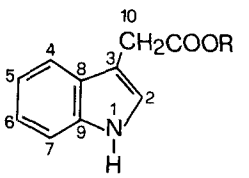
Abstract. From the culture filtrates of *Pseudomonas amygdali* the methyl ester of indole-3-acetic acid (IAA), a product of indole-3-acetic acid metabolism which has the same auxin activity as the free acid, has been isolated. This is the first report of its occurrence as a microbial metabolite.
Key words. Pseudomonas amygdali; almond hyperplastic bacterial canker; auxins; indole-3-acetic acid; indole-3-acetic acid methyl ester.

Pseudomonas amygdali Psallidas and Panagopoulos is a pathogen of almond trees[1-3], which produces high levels of indole-3-acetic acid (IAA) and cytokinins in culture[4,5]. The accumulation of these two groups of plant growth substances in culture seems to be correlated with the virulence of the producing organism[6]. However, the IAA isolated from organic acid (EtOAc) extracts did not account for all the indoles present in the bacterial culture filtrates[4,6]. In fact, TLC analysis (silica gel, Merck Kieselgel 60 F_{254}, 0.25 mm, using chloroform-methanol 6:4, v:v and chloroform-ethyl acetate-methanol 2:2:1, v:v:v as solvent systems) of the EtOAc extracts showed the presence, in addition to IAA, of other UV-absorbing bands which reacted positively with reagents staining indole and indole-3-substituted derivatives[7,8].

The important role of auxins in development of the disease symptoms in plants[6] prompted us to reinvestigate the nature of the indoles other than IAA produced by *P. amygdali*. Here we report on the isolation and identification of the methyl ester of IAA, which was found to be the main indole present in addition to IAA in the culture filtrate of *P. amygdali*.

Materials and methods

Auxin production, extraction and purification. P. amygdali strain NCPPB 2610 was grown as previously reported[4] and culture filtrates (3.0 l) were extracted at pH 2.5 with ethyl acetate. Crude acid extract (861 mg) was fractionated on a 2.5 × 70 cm Sephadex LH-20 (Pharmacia 25–100 μm) column; eluent: chloroform-methanol (6:4, v:v); elution rate: 3 ml/min; fraction volume: 3 ml. The fractions containing the IAA-methyl ester (**2**) were combined and evaporated under reduced pressure. The residue (53.1 mg) was further purified by preparative TLC (silica gel, Merck Kieselgel F_{254}, 0.5 mm, eluent chloroform-ethyl acetate-methanol 2:2:1, v:v:v), to give **2** as a pure oil (24 mg, 8.0 mg/l), as shown by its TLC analysis on silica gel, (using the solvent systems above cited but also chloroform-*iso*-propanol 95:5, v:v) and by reverse phase chromatography (Whatman, Stratocrom C-18 SIF_{254}, 0.20 mm, using water-acetonitrile 1:1, v:v as solvent system).



1 R=H
2 R=Me

Auxin identification. The UV spectra were recorded in methanol on a Perkin-Elmer 550 S spectrophotometer. The natural compound was essentially identified by ^{1}H- and ^{13}C-NMR spectra in CD_3OD at 250 and 62.50 MHz, respectively, on a Bruker AC 270 spectrometer, using the same solvent as for the internal standard. EI-MS were performed at 70 eV on a Kratos MS-80 spectrometer.

Bioassay. Auxin activity was assessed by the wheat coleoptile straight growth assay. The method described by Nitsch and Nitsch[9] was followed with minor modifications. Compounds were dissolved in a minute amount of methanol and brought to the required concentration with posphate-citric buffer (10 mM K_2PO_4, 5 mM citric acid, 2% sucrose, pH 5). The buffer containing the same amount of methanol (0.1%) was used for comparison.

Results and discussion

The structure of the auxin metabolite (**2**), isolated together with IAA (**1**) from the culture filtrates of *P. amygdali*, was mainly deduced from its spectral properties. In fact, the UV spectrum of **2** showed a fine structured maximum at 278 nm (log $\varepsilon = 3.44$), a typical absorption value of indole-3-substituted derivatives[10]. The true chemical nature of **2** was revealed by the analysis of its ^{1}H- and ^{13}C-NMR spectra (tables 1

Table 1. ^{1}H-NMR data (CD_3OD, at 250 MHz) of indole-3-acetic acid (**1**) and its corresponding methyl ester (**2**). Chemical shifts are in δ-values (ppm) from TMS

	1	2
H-2	7.12 *s*	7.13 *s*
H-4*	7.59 *dd*	7.50 *dd*
H-5*	6.95 *ddd*	7.00 *ddd*
H-6*	7.04 *ddd*	7.09 *ddd*
H-7*	7.28 *dd*	7.33 *dd*
2H-10	3.06 *s*	3.75 *s*
CH_3O	-	3.66 *s*

J (Hz): **1**, **2**: 4, 5 = 5, 6 = 6, 7 = 8.0; 4, 6 = 5, 7 = 1.5.
*Attributions made in agreement with data reported for derivatives of indole[15].

Table 2. ^{13}C-NMR data (CD_3OD at 62.5 MHz) of indole-3-acetic acid (**1**) and its corresponding methyl ester (**2**). Chemical shifts are in δ-values (ppm) from TMS

	1	2
C-2*	124.6 *d*	124.7 *d*
C-3*	108.9 *s*	108.6 *s*
C-4*	119.8 *d*	119.9 *d*
C-5*	122.5 *d*	122.5 *d*
C-6*	119.4 *d*	119.3 *d*
C-7*	112.2 *d*	112.3 *d*
C-8*	128.7 *s*	128.6 *s*
C-9*	138.0 *s*	138.0 *s*
C-10	31.9 *t*	31.9 *t*
C = O	176.5 *s*	174.8 *s*
CH_3O	-	52.3 *q*

*Assigned also by comparison with data reported for indole-3-substituted derivatives[16].

and 2, respectively). In fact, compared to IAA (**1**) **2** differed significantly only in the presence of the signals due to the ester methoxy group which appeared as a singlet at δ 3.66 and as a quartet at δ 52.3 in the ^{1}H- and ^{13}C-NMR spectra, respectively.

In addition, the electron impact mass spectrum exhibited the molecular ion at *m/z* 189 (15%) and a prominent peak at *m/z* 130, in agreement with the fragmentation pathway described for indole-3-substituted derivatives[11].

Moreover, natural **2** showed the same chromatographic behaviour as the methyl ester obtained by treating an authentic sample of IAA (purchased from Fluka Chemie AG, Buchs, Switzerland) with ethereal diazomethane, also when co-chromatographed, using TLC on silica gel (eluents: chloroform-*iso*-propanol, 95:5, v:v; benzene-acetone, 8:2, v:v and methylene chloride) and on reverse phase chromatography (water-acetonitrile 1:1, v:v). Synthetic **2** exhibited the same spectroscopic data (UV, ^{1}H and ^{13}C-NMR and EIMS) as the natural metabolite.

Finally, when equimolar solutions of IAA and of its methyl ester were tested for auxin activity in the wheat coleoptile straight growth assay[9] they showed, as expected[12], the same activity.

Esters of indole-3-acetic acid are widespread in plants and have been discussed as storage forms of auxin[12]. However, to the authors' knowledge, IAA methyl ester has never previously reported as a microbial metabolite. The function of the methyl ester of IAA in *P. amygdali* and in its interaction with the host plant is still unknown. However, since a common basis for tumorigenesis in plant-pathogen interaction is the alteration of phytohormone levels and their balance in the infected tissues[13], this active product of IAA metabolism along with IAA[6] may play an important role in the development of hyperplastic cankers on almond. Moreover, it cannot be excluded that the IAA methyl ester, as is the case for IAA lysine conjugates in the closely-related bacterium *P. syringae* pv. *savastanoi*[14], might play a role in virulence through regulating the pool size of IAA.

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