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α-*N*-ACETYL-INDOLE-3-ACETYL-ε-L-LYSINE: A METABOLITE OF INDOLE-3-ACETIC ACID FROM *PSEUDOMONAS SYRINGAE* PV. *SAVASTANOI*

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Key Word Index—*Pseudomonas syringae* pv. *savastanoi*; olive and oleander knot disease; auxin; phytohormones; indole-3-acetic acid derivatives.

Abstract – A product of indole-3-acetic acid (IAA) metabolism having an auxin-like activity has been isolated from liquid cultures of *Pseudomonas syringae* pv. *savastanoi*. By spectral data and chemical correlations the compound has been identified as α-*N*-acetyl-indole-3-acetyl-ε-L-lysine (Ac-IAA-Lys). The IAA-derivative was detected in culture filtrates of oleander strains but not in culture filtrates of olive strains. The physiological effects of Ac-IAA-Lys on hypocotyl elongation in wheat, leaf chlorosis in oleander and bean and the hypertrophic response of potato tuber discs were compared with those of IAA. The results indicated that Ac-IAA-Lys was approximately 60% less active than IAA.

INTRODUCTION

It has been found that biologically active compounds are produced in culture by *Pseudomonas syringae* pv. *savastanoi* (pv. *savastanoi*), the causal agent of olive and oleander knot disease. The main active compounds were identified as indole-3-acetic acid (IAA, **1**) [1] and various cytokinins [2–4]. The phytohormone IAA is formed by pv. *savastanoi* from L-tryptophan via indole-3-acetamide [5, 6] and it is further converted into water-soluble metabolites. One of these metabolites has been identified as indole-3-acetyl-ε-L-lysine (IAA-Lys, **2**) [7]. The present paper reports on the isolation, purification and structural elucidation of a new metabolite of IAA from pv. *savastanoi*, which is a derivative of IAA-Lys.

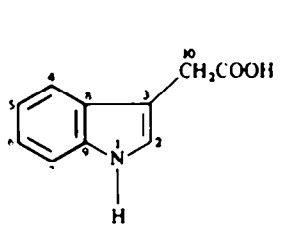
RESULTS AND DISCUSSION

The material obtained from the lyophilization of 5-day-old culture filtrates of different strains of pv. *savastanoi* was partioned between aqueous acid and ethyl acetate. The residues left by organic extraction was redissolved in a small volume of water and tested for auxin activity on wheat hypocotyls. Preparative TLC of the active extracts yielded IAA (**1**) from the extracts of all wild-type pv. *savastanoi* strains and another apparently homogeneous substance (**3**) from the extracts of the wild-type oleander strains. IAA-deficient mutants did not accumulate either of the two compounds.

Compound **3** had a lower R_f than **1** on normal phase TLC. On reverse phase TLC, the chromatographic pattern was reversed.

Purified **3** caused chlorosis in oleander and bean leaves, stimulated wheat hypocotyl elongation and induced hypertrophy in potato tubers. It was, however, 60% less effective in these bioassays than extracted or authentic IAA.

Compound **3** had UV absorption maxima at 288, 279 and 220 nm in agreement with those reported for indole-3-acetic acid derivatives [8]. Its ^{1}H NMR spectrum (Table 1) showed two singlets at δ7.16 and 3.63 respect-



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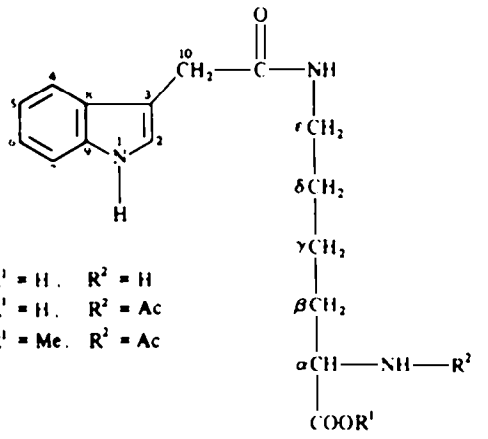

2 $R^1 = H$, $R^2 = H$
3 $R^1 = H$, $R^2 = Ac$
4 $R^1 = Me$, $R^2 = Ac$

Table 1. ^{1}H NMR spectral data of compounds 3 and 4 (270 MHz, TMS as internal standard)

H	3 (CD_3OD)	4 ($CDCl_3$)
1		8.32 *s* (*br*)
2	7.16 *s*	7.14 *d*
4*	7.52 *dd*	7.54 *dd*
5*	7.01 *ddd*	7.15 *ddd*
6*	7.09 *ddd*	7.24 *ddd*
7*	7.34 *dd*	7.40 *dd*
10	3.63 *s*	3.72 *s*
α†	4.21 *dd*	4.49 *ddd*
β†	1.78 *ddt*	1.68 *ddt*
β'†	1.63 *ddt*	1.62 *ddt*
γ† (2H)	0.83 *m*	1.21 *m*
δ† (2H)	1.46 *m*	1.35 *m*
ε†	3.15 *t*	3.19 *ddt*
ε'†		3.13 *ddt*
α-NH		6.19 *d*
ε-NH		5.76 *t*
MeCO	1.94 *s*	1.99 *s*
MeO		3.71 *s*

J (Hz): 3, 4: 4,5 = 5,6 = 6,7 = 8.1; 4,6 = 5,7 = 1.5; α,β = 4.8; α,β' = 8.1; β,β' = 15.0; β,γ = β',γ = 7.5; ε,δ = ε',δ = 7.0; 4: 1,2 = 2.5 ε,ε' = 9.9; α,NH = 8.1; ε,NH = ε',NH = 5.9.

* and † assigned also in comparison to data reported in literature for derivatives of indole [9] and lysine [10], respectively.

Table 2. ^{13}C NMR data of compound 3 (67.88 MHz, CD_3OD as solvent and internal standard)

C		C	
2†	124.88 *d**	α‡	56.20 *d**
3†	109.53 *s*	β‡	33.97 *t*
4†	119.94 *d*	γ‡	24.14 *t*
5†	122.55 *d*	δ‡	33.55 *t*
6†	119.31 *d*	ε‡	40.40 *t*
7†	112.31 *d*	HNC=O	174.85 *s*
8†	128.56 *s*	HOC=O	179.27 *s*
9†	138.13 *s*	C=O	172.50 *s*
10	30.08 *t*	Me	22.70 *q*

*By SFORD.

† and ‡ attributed also in comparison to the data reported in literature for derivatives of indole [12] and lysine [13], respectively.

ively, two double doublets at δ7.52 and 7.34 and two doublets of double doublets at δ7.09 and 7.01 consistent with the presence of a monosubstituted indole moiety. In fact, the proton resonance pattern was very close to that reported for **1**. [9]. In addition, a double doublet was observed at δ4.21 and a triplet at δ3.15. These latter signals, together with the complex systems present at δ1.78 and 1.63 (both doublets of double triplets) and the two multiplets recorded at δ1.46 and 0.83, indicated, also by comparison with literature data [10], the presence of a lysine residue. Moreover, a singlet, typical of an acetyl group, was observed at δ1.94.

The fast atom bombardment (FAB) mass spectrum of **3** gave a pseudo molecular ion at *m/z* 346 and a fragmentation peak at *m/z* 130 characteristic of a 3-substituted indole derivative [11].

The ^{13}C NMR spectrum of **3** (Table 2) indicated that **3** was an IAA-lysine conjugate. This finding was in agreement with the data reported previously for indole [12] and lysine [13] derivatives. However, the two lysine amino groups were not free bases. In fact, **3** was ninhydrin insensitive. This was further evidenced by the two singlets at δ174.85 and 172.50, a characteristic resonance for the ketonic group of amides; the signal of the carboxylic carbon appeared as a singlet at δ179.27. Consequently, when treated with ethereal diazomethane, the new IAA-derivative gave the corresponding monomethyl ester (**4**).

Compared to that of **3** the ^{1}H NMR spectrum of **4** (Table 1) recorded in $CDCl_3$ showed the presence of a singlet at δ3.71 due to the methoxyl group. Moreover, the signals of the three NH groups were observed. The broad singlet at δ8.32 was assigned to the NH of the indole system. The doublet at δ6.19 and the triplet at δ5.76 were attributed by ^{1}H NMDR to the α-NH and to the ε-NH groups of the lysine residue, respectively. The signals due to the α-CH and to the ε-CH_2 of the same moiety appeared to be a more complex system (doublet of a double doublet and two doublets of a double triplet, respectively) in the spectrum of **4** with respect to the pattern observed for **3**. These multiplicities arose from the further coupling of each proton described with the corresponding vicinal NH group.

In the EIMS of **4** the molecular ion at *m/z* 359 and the typical fragmentation peak at *m/z* 130 [11] were observed.

The presence in **3** of a lysine residue was confirmed by amino acid analyses performed on the acid hydrolysate of **3**. The chromatographic pattern showed only one peak which was identified as lysine by its retention time. A portion of the same sample was reacted with L-lysine saccharopine dehydrogenase (Sigma Chemical Co.) by the procedure of Nakatani *et al.* [14]. After a 30 minute incubation the yield of saccharopine was 95% thus indicating that the L isomer of lysine was present in **3**. A similar yield (94%) was obtained when the same procedure was applied to an authentic sample of L-lysine; conversely no reaction was observed when D-lysine was used as substrate.

Compound **3** was clearly identified as α-*N*-acetyl-indole-3-acetyl-ε-L-lysine (Ac-IAA-Lys) by direct comparison of its spectral properties (^{1}H NMR and UV), optical rotation and R_f in three different TLC systems with those possessed by the synthetic acetyl derivative prepared from indole-3-acetyl-ε-L-lysine (IAA-Lys, **2**).

The procedure used to extract **3** did not lead to the formation of artifacts. In fact, when the same sequence of steps was applied to IAA-Lys it remained unchanged in the aqueous solution, while any other compounds, including **3**, were observed in the ethyl acetate phase.

IAA-Lys is a known metabolite previously isolated from culture filtrate of pv. *savastanoi* [7]. It represents a product of IAA metabolism but its function in the bacterium is unknown, although it has been suggested that the conversion of IAA into IAA-Lys could be a mechanism for reducing the biological activity of IAA.

In any case, under our experimental conditions, we were unable to detect the presence of IAA-Lys in the extracts of all pv. *savastanoi* strains used throughout this research work. Moreover, we isolated the acetyl derivative of IAA-Lys from culture filtrates of pv. *savastanoi* oleander

strains but not from olive strains. Time course experiments indicated that the appearance of compound 3 in the culture medium followed the synthesis of IAA. In fact, only IAA was detected in the 3-day-old culture filtrates of strain ITM 519. After that time, the amount of IAA increased progressively with growth of bacteria as did compound 3 after 5, 7 and 10 days of incubation.

Finally, we observed that strains ITM 519 and its IAA-deficient mutants (IAA-non-producers) grown in Woolley's medium supplemented with 0.03% of IAA did not accumulate detectable amounts of compound 3. This could indicate that compound 3 synthesis is inhibited by large amounts of IAA. This was not observed in the case of IAA-Lys [7].

The effect of IAA and compound 3 on oleander leaves (chlorotic halos are formed in nature around knots developing on oleander leaves) could be due to the production of ethylene stimulated by the presence of an excess of IAA. Studies are under way to clarify this point.

EXPERIMENTAL

General methods. Analytical and prep. TLC: silica gel (Merck, Kieselgel 60 F_{254}, 0.25 and 2.0 mm respectively) and reversed phase ODS (Stratocrom C-18 Whatman 0.2 mm) plates. The spots were visualized by exposure to UV radiation or I_2 vapour. Solvent systems: (A) $CHCl_3$–MeOH (1.5:1); (B) $CHCl_3$–*iso*-PrOH (9:1); (C) *n*-BuOH–HOAc–H_2O (4:1:1.6); (D) H_2O–MeCN (1.5:1); FAB/MS 50 (Kratos Ltd): sample dissolved in glycerol/thioglycerol on a copper probe tip and inserted into the source at 10^{-5} torr pressure of Ar. The sample was bombarded with Ar atoms f 6–8 kV energy and the spectrum was recorded on UV paper.

Culture. Pathovar *savastanoi* (ITM* 519 from oleander) was grown in 6.8 l of Woolley's medium [15] using 17 × 1 l. flasks on a rotary shaker for 5 days at 26°. Strain ITM 519, strains NCPPB* 639, PVBa* 225 and ITM 317 from olive, strains PVBa 219, and NCPPB 640 from oleander, and the respective IAA-deficient mutants [16] were used in the comparative studies. Bacteria were removed by centrifugation (12 000 *g*) and discarded. The culture filtrate (6.5 l.) was lyophilized. The residue was dissolved in 650 ml H_2O and the pH of the soln adjusted to ~ 2 using 1 M HCl. The aq. soln was then extracted with EtOAc (4 × 300 ml); the combined organic extracts were dried (Na_2SO_4) and then evaporated under red. pres. to give a crude oily residue (91.1 mg). For time course experiments, the culture filtrates of strain ITM 519 were collected 3, 5, 7 and 10 days after inoculation.

Bioassay. Three bioassays were used routinely. (i) Hypertrophy of potato tuber discs was determined by the method described by Sakai [17]. Test solns were applied in 10 μl Me_2CO to filter paper discs (6 mm diameter) and air dried. The discs were then transferred onto the upper surface of potato tuber discs (3 mm × 8 mm diam), moistened with sterile water, and incubated at 21°. (ii) Elongation in wheat hypocotyl. Seeds of *Triticum vulgare* L. were soaked in tap H_2O overnight and allowed to germinate at 28° for 48 hr in the dark. Then, 3 mm long sections from 2 cm long hypocotyls were isolated 3 mm below the tip, transferred onto filter paper and moistened with test solns in Petri dishes. Length measurements were taken after 24 hr. (iii) Leaf chlorosis. Three 10 μl droplets of the test soln were deposited on the ventral surface of a detached oleander leave, which was scratched with a needle, passing through each droplet. The treated leaves were placed in a desiccator and the pressure was reduced (× 5) to ~ 80 mm Hg for 2 min. This treatment resulted in the complete infiltration of the test droplets into the leaves, which were then removed from the desiccator placed in a large Petri dish and incubated at 25° under light. In addition to oleander, bioassays were also made on leaves of bean using the pin-prick method [18]. The test solns were bioassayed in comparison to authentic IAA at concentrations of 10^{-3} to 10^{-5} M.

Auxin separation. The crude EtOAc extracts (91.1 mg) active on wheat hypocotyls were purified by prep. TLC (silica gel, eluent A), yielding two UV absorbing bands. The upper zone was scraped off and eluted with MeOH. On evaporation of the solvent, an oily residue (1, 26 mg) was given off. From the lower band treated in the same way, a chromatographically homogeneous (TLC: silica gel, eluents A and C; reversed phase, eluent D) oil-like substance (3) was obtained (28.7 mg). Pure 3 showed: $[\alpha]_D^{25} = +8.42$ (*c* 1.19; MeOH); UV λ_{max}^{MeOH} nm: 288, 279, 272 and 220 (log ε: 3.54, 3.61, 3.58 and 4.33, respectively); ^1H amd ^{13}C NMR see Tables 1 and 2 respectively.

Methyl ester of 3 (4). Compound 3 (14.6 mg) was dissolved in MeOH (1 ml) and treated with an ethereal soln of CH_2N_2 (2.0 ml) at room temp. for 2 days. The reaction was stopped by evaporation of the solvent under a stream of N_2. The crude residue (11.3 mg) was purified by prep. TLC (silica gel plates, eluent B), yielding chromatographically pure 4 (7.5 mg, 51%); UV λ_{max}^{EtOH} nm: 288, 279, 274 and 220 (log ε: 3.33, 3.48, 3.45 and 4.12, respectively); IR $\nu_{max}^{CHCl_3}$ cm $^{-1}$: 3400 (NH), 1720 and 1650 (C=O); ^1H NMR see Table 1; MS (EI, 70 eV), *m/z* (rel. int.): 359 $[M]^+$ (100), 344 (2.5), 328 (0.3) and 130 (99).

Hydrolysis of 3. Compound 3 (0.62 mg, 1.79 μmol) and 6 M HCl (1 ml) were heated at 110° in a sealed vial for 24 hr; after cooling, the hydrolysate was dried under vacuum and analysed for lysine content by chromatographic and enzymatic procedures. TLC (eluent C) showed the presence of lysine as the only ninhydrin sensitive spot; quantitative amino acid analyses gave 1.72 μmol of lysine (96% yield). Enzymatic determination, carried out using the method described by Nakatani *et al.* [14], gave 1.70 μmol of L-lysine (95% yield).

Acetylation of 2. Compound 2 (4.0 mg) was acetylated with a mixture of Ac_2O (400 μl) and NaOAc (4 mg). The reaction was performed at 0° (with stirring) in order to avoid acetylation of the indole nitrogen. After 2 hr, the reaction mixture was poured into ice water. The aq. soln was then acidified, its pH adjusted to ~ 2 with 1 M HCl and extracted with EtOAc (3 × 10 ml). The combined organic extracts were dried (Na_2SO_4) and evaporated under red. pres. The residue was purified by prep. TLC according to the conditions reported for 3. The pure oil obtained (3 mg, 75%) showed: $[\alpha]_D^{25} = +10.1$ (*c* 0.36; MeOH); UV λ_{max}^{MeOH} nm: 288, 279, 272 and 220 log ε: 3.53, 3.61, 3.59 and 4.31); ^1H NMR: identical to that reported for 3 (Table 1). The monoacetyl of 2 showed the same R_f as 3, according to TLC analysis on silica gel (eluent A and C) and on reverse phase (eluent D) plates, also by co-chromatography.

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*ITM, Collection of Istituto Tossine e Micotossine da Parassiti Vegetali del C.N.R.; PVBa Collection of Dipartimento di Patologia Vegetale, Universita' di Bari; NCPPB National Collection of Plant Pathogenic Bacteria, Harpenden, England.

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