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A CYTOKININ FROM THE CULTURE FILTRATE OF *PSEUDOMONAS SYRINGAE* PV. *SAVASTANOI**

GIUSEPPE SURICO, ANTONIO EVIDENTE†, NICOLA S. IACOBELLIS and GIACOMINO RANDAZZO†

Istituto Tossine e Micotossine da Parassiti Vegetali del C.N.R., Via Amendola 197/F, 70126 Bari, Italy; †Dipartimento di Chimica Organica e Biologica, Università di Napoli, Via Mezzocannone 16, 80134 Napoli, Italy

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Key Word Index—*Pseudomonas syringae* pv. *savastanoi*; olive knot; hypertrophy; cytokinins; phytohormones; adenine derivatives.

Abstract—The structure of a new cytokinin, isolated from the culture filtrate of *Pseudomonas syringae* pv. *savastanoi*, is assigned on the basis of spectroscopic data including its tetracetyl derivative and comparison with related adenine derivatives. It was identified as 6-(4-hydroxy-1,3-dimethylbut-*trans*-2-enylamino-9-β-D-ribofuranosyl)purine.

INTRODUCTION

Olive knot, incited by *Pseudomonas syringae* pv. *savastanoi*, is one of the most important diseases of olive in the south of Italy. The disease, which also occurs on oleander, privet and ash, is characterized by the formation of galls on young stems and, less frequently, on other organs of the plants. Previous investigations [1, 2] have shown that cytokinins accumulated in the culture medium of pathogenic isolates of pv. *savastanoi*. Ethyl acetate extracts of culture filtrate stimulated the growth of olive callus as well as tobacco callus. Several active compounds were separated by TLC but their identities have not been determined.

In the present study, we report our results on the characterization of a new cytokinin isolated from the culture medium of pv. *savastanoi* together with the identification of two other known cytokinins [3].

RESULTS AND DISCUSSION

Preparative TLC (silica gel) was a valuable first step in the recovery of the cytokinins from the basic organic extracts of a large volume of medium as all the biological activity was localized in a single UV absorbing band with a zeatin-like R_f value. The second purification stage performed by analytical TLC (silica gel) allowed the separation of this band into two active zones of R_f 0.66 (mixture A) and 0.73 (mixture B), respectively. The two fractions were further purified to give homogeneous substances by reverse phase TLC (Stratocrom C-18). Both mixtures each yielded two UV absorbing bands: A1 (R_f 0.51, zeatin region), A2 (R_f 0.66, zeatin riboside region) and B1 and B2 whose R_f values did not correspond to any of the standards used.

The novel nature of compounds B1 and B2 was confirmed by HPLC analysis [R_t 7.93 and 8.40 min, respectively, using 0.01 M ammonium carbonate–methyl cyanide (8:2), as eluent, at a flow rate of 0.5 ml/min] co-injected with reference compounds. The use of the same procedure allowed the identification of compounds A1 and A2 (R_t 4.25 and 6.25 min, respectively) as *trans*-zeatin (**1**) and *trans*-zeatin riboside (**2**), respectively. Compound B1 is under investigation, while compound B2 has been identified as 6-(4-hydroxy-1,3-dimethylbut-*trans*-2-enylamino)-9-β-D-ribofuranosylpurine (**3**).

Pure **3** exhibited an UV spectrum characteristic of N^6-9-disubstituted adenine derivatives. The ^{1}H NMR spectrum (Table 1) was consistent with a zeatin riboside-type structure. The proton shifts were assigned by integration, multiplicity and ^{1}H NMDR. The distinction between the resonances of the protons of the purine system was made by comparison with spectroscopic data available from the lit. [4]. Furthermore, a doublet was present at δ1.35 due probably to a secondary methyl group attached to the 4-hydroxyisopentenyl side chain. Accurate inspection of the spectrum showed the different multiplicity of the signal due to H-2″, a broad doublet at δ5.53, as compared with the broad triplet attributed to the same proton in the spectra of **1**, **2** and the isopentenyladenosine (**4**) [3]. The signal of the proton bonded to C-1″ was observed as a large multiplet at δ5.27. Examination of the spectrum (Table 1) of the tetracetyl derivative of **3** (**5**), recorded at 500 MHz, in deuterochloroform, confirmed the above observations. Irradiation of the broad multiplet at δ5.24 (H-1″) converted both doublets at δ1.37 (Me-6″) and 5.77 (HN-10) into two singlets. This irradiation also simplified the signal at δ5.47 (H-2″) into a broad singlet. These results suggest for structure **3** the new cytokinin.

The ^{13}C NMR spectrum (Table 2) was in agreement with the proposed structure. The carbon shifts were assigned by proton noise decoupled and by off-resonance decoupled spectra. The assignment of the quaternary carbons of the purine rings and of the carbons of the ribose moiety was made by comparison with reported lit. data [5].

The above findings were supported also by the occurrence of the pseudo molecular ion at m/z 366 in the fast atom bombardment (FAB) mass spectrum of **3**. The fragmentation peaks, observed at m/z 349 $[M-NH_2]^+$,

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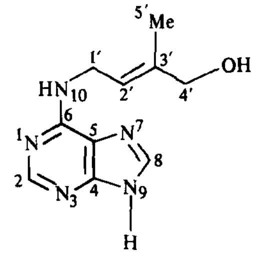

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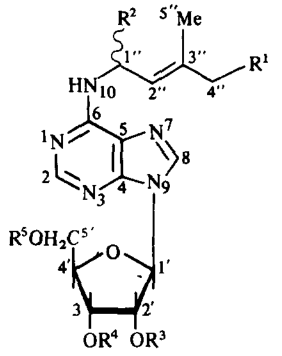

	R^1	R^2	R^3	R^4	R^5
2	OH	H	H	H	H
3	OH	6″Me	H	H	H
4	H	H	H	H	H
5	OAc	6″Me	OAc	OAc	OAc

307 $[M-C_3H_6O]^+$, 215 [free base $-H_2O]^+$, 200 [215 $-$ Me$]^+$, 148 [free base $-C_5H_9O]^+$, 136 [adenine + H$]^+$ and 108 [adenine $-$ HCN$]^+$, are characteristic of 6-alkylaminopurines [6–8]. The peak at *m/z* 276 $[M-89]^+$, which arises from a fragmentation process involving the sugar moiety, indicates the presence of a ribosyl residue [9]. In addition, the EI mass spectrum of **5** showed a $[M]^+$ at *m/z* 533. The peaks at *m/z* 259, 199, 139 and 97 were very similar to the fragmentation scheme reported for triacetyl pentose [10]. Moreover, acid hydrolysis of **3** yielded β-D-ribose, as observed by co-chromatography with a reference sample in three different TLC systems.

Final evidence to unequivocally assign the structure to **3** was obtained from comparison of its ^{1}H NMR spectrum with those of **2** and **4** recorded under the same conditions (Table 1). In particular, the presence in **3** of the ribosyl moiety appeared from the proton pattern (chemical shifts and coupling constants) shown by the pentose residue of the three nucleosides.

The stereochemistry of the double bond present in the side chain was also established. Evidence for the presence of a 4-hydroxy-1″,3″-dimethyl-2-*trans*-enyl residue bonded to N-10 of **3** was obtained. The larger value of the allylic coupling constant between H-2″ and H-4″ (1.8 Hz) than the corresponding one due to H-2″ and H-5″ coupling (1.2 Hz) was in agreement with the commonly observed data for acyclic compounds in that $|J_{cisoid}| > |J_{transoid}|$ [11, 12]. This result was supported by the downfield shift (Δδ0.15) observed for the signal of H-2″ in the spectrum of **3** as compared with that of **4**. Unequivocal evidence was obtained from the ^{1}H NOE difference spectrum of **5**. A clear effect was observed at δ4.46 (CH_2-4″) by homonuclear selective decoupling at δ5.47 (H-2″). The determination of the absolute configuration of Me-6″ is under investigation.

Table 1. ^{1}H NMR spectral data of compounds **2–4** (270 MHz) and **5** (500 MHz) (δ-values)

H No.	**2** (CD_3OD)	**3** (CD_3OD)	**4** (CD_3OD)	**5*** ($CDCl_3$)
2†	8 23 (*s*)	8 22 (*s*)	8.22 (*s*)	8.37 (*s*)
8†	8.24 (*s*)	8.24 (*s*)	8.24 (*s*)	7.87 (*s*)
10	—	—	—	5.77 (*br d*)
1′	5.94 (*d*)	5.94 (*d*)	5.94 (*d*)	6.16 (*d*)
2′	4.73 (*dd*)	4 73 (*dd*)	4.74 (*dd*)	5.91 (*dd*)
3′	4.31 (*dd*)	4.31 (*dd*)	4.31 (*dd*)	5.66 (*dd*)
4′	4.16 (*ddd*)	4.17 (*ddd*)	4 16 (*ddd*)	4.43 (*ddd*)
5′A	3 88 (*dd*)	3.88 (*dd*)	3.89 (*dd*)	4.42 (*dd*)
5′B	3.74 (*dd*)	3.74 (*dd*)	3 74 (*dd*)	4.38 (*dd*)
1″	4.27(2H) (*m*)	5.27 (*m*)	4.19 (2H) (*m*)	5 24 (*m*)
2″	5.64 (*br t*)	5.53 (*br d*)	5.38 (*br t*)	5.47 (*br d*)
4″	3.97 (2H)(*br s*)	3.94(2H) (*br s*)	1.76(3H) (*br s*)	4 46(2H) (*br s*)
5″(3H)	1 78 (*br s*)	1 77 (*br s*)	1 76 (*br s*)	1.80 (*br s*)
6″(3H)	—	1 35 (*d*)	—	1.37 (*d*)

J (Hz) **2–5**: 5′A, 5′B = 12 5; **2–4**: 2′, 3′ = 5.2; **3, 4**: 3′, 4′ = 4′, 5′A = 4′, 5′B = 2.6; **2, 5**. 3′, 4′ = 4′, 5′A = 4′, 5′B = 2 4; **2**: 1′, 2′ = 6.4; 1″, 2″ = 7.0; 2″, 4″ = 1.5; 2″, 5″ = 1.1, **3**. 1′, 2′ = 5 3; 1″, 2″ = 8.8; 1″, 6″ = 6.7; 2″, 4″ = 1.8; 2″, 5″ = 1.2; **4**: 1′, 2′ = 6.3; 1″, 2″ = 7 0; **5**: 1′, 2′ = 2′, 3′ = 5.5; 1″, 2″ = 8.2; 1″, 6″ = 6.7; 1″, 10 = 7.9; 2″, 4″ = 1.8; 2″, 5″ = 1.2.

*Signals of the four acetyl groups were observed at δ2 08 (*s*), 2.09 (*s*), 2.13 (*s*) and 2 14 (*s*), respectively

†Assigned in agreement with lit. data [4].

Table 2. ^{13}C NMR chemical shifts of compound 3 (67.88 MHz, CD_3OD, using the same solvent as int. standard) (δ-values).

C No		C No.	
2	153.6 (*d*)†	4′*	88.2 (*d*)
4*	149.9 (*s*)	5′*	63.5 (*t*)
5*	116.7 (*s*)	1″	45.5 (*d*)
6*	157.1 (*s*)	2″	127.7 (*d*)
8	141 4 (*d*)	3″	138.3 (*s*)
1′*	91.3 (*d*)	4″	68.0 (*t*)
2′*	72.7 (*d*)	5″	21.8 (*q*)
3′*	75.4 (*d*)	6″	14.1 (*q*)

*Assignments given by comparison with available reference compounds [5].

†By SFORD multiplicity.

EXPERIMENTAL*

General methods. Analytical and prep. TLC were carried out on silica gel (Merck, Kieselgel 60 F_{254}, 0.25 and 2.0 mm, respectively), on reversed phase ODS (Stratocrom C-18 Whatman, 0.2 mm) and on cellulose (Merck, DC-Fertigplatten, 0.1 mm) prepared plates The spots were visualized by exposure to UV and by spraying with a 10% H_2SO_4 soln in MeOH or with a 1% $AgNO_3$ soln in 0.65 M NH_4OH and then heating at 105°. Solvent systems. (A) *n*-BuOH–HOAc–H_2O (4:1:1.6); (B) H_2O–EtOH (1.5·1); (C) $CHCl_3$–*iso*-PrOH (9:1); (D) H_2O–EtOH (19:1). HPLC analyses were performed on a Merck Hibar LiChroCart column (4.0 mm i.d. × 25 cm) using a spectrophotometric detector at 262 nm; the mobile phase was 0.01 M $(NH_4)_2CO_3$–MeCN (9·1) at a flow of 3 ml/min. FAB MS were determined from samples dissolved in glycerol–thioglycerol on a Cu probe tip and inserted into the source at 10^{-5} Torr pressure of Ar. The sample was bombarded with Ar atoms of 6–8 kV energy and spectra were recorded on UV paper. An authentic sample of zeatin (Z), zeatin mixed isomers, dihydrozeatin (diHZ), 9-β-D-ribofuranosylzeatin (ZR), 9-β-D-ribofuranosyldihydrozeatin (diHZR), 6-benzylaminopurine (BAP), isopentenyladenosine (iPA), 2-methylthioisopentenyladenosine (2MSiPA), 2-methylthioisopentenyladenine (2MSiP), 2-methylthio-9-β-D-riboruranosylzeatin (2MSZR) and 2-methylthio-9-β-D-ribofuranosyl-*cis*-zeatin were used as standards.

Cytokinin production and extraction. Pv *savastanoi* (NCPPB† 640) was grown with slight agitation in Woolley's medium [13] for 10 days at 26°. Bacteria were removed by centrifugation (5000 *g*, 10 min) and filtration (Millipore 45 μm) and discarded. The culture filtrate (27.5 l.) was lyophilized and the residue dissolved in a vol. of H_2O equal to 10% of the bacterial suspension and centrifuged. The soln was adjusted to pH 8.5 with 1 M KOH and extracted with EtOAc (5 × 1.65 l.) The organic combined extracts were dried (Na_2SO_4) and evaporated under red. pres at 30° Cytokinin activity was determined by the cucumber cotyledon bioassay based on ref. [14] and expressed as mg zeatin equivalent/l of culture medium.

Cytokinin fractionation. Crude EtOAc extract (423.0 mg), with a cytokinin activity of 5.3 mg zeatin equivalents/l. of culture medium, was purified by prep. TLC (silica gel, eluent A) yielding a fraction, UV absorbing, which showed a R_f value similar to zeatin. The band was scraped from the plates and eluted with EtOH; evaporation of solvent gave an oily residue (325 mg). This was separated by TLC (analytical silica gel, eluent A). After development, two zones were located by brief exposure to UV, and were scraped off and eluted with EtOH. Evaporation of solvent gave the following residues: A (from lower zone, 35.3 mg) monitored by TLC (reverse phase, eluent B) shown to contain **1** (Z) and **2** (ZR); B (from upper zone, 33.8 mg) formed from two unknown compounds with cytokinin activity. The two mixtures were both further purified by reverse phase TLC (eluent B). From A was obtained **1** separated from **2**. Residue B furnished two cytokinins, 6.4 and 7.3 mg B1 and B2 (**3**), respectively, both homogeneous by TLC and HPLC analysis. Pure **3** showed· UV $\lambda_{\text{max}}^{\text{EtOH}}$ nm: 270 ($\varepsilon = 8.01 \times 10^3$); ^{1}H and ^{13}C NMR· see Tables 1 and 2, respectively.

Tetracetyl **3**. To prepare the peracetyl derivative of **3** the compound (2 mg) was dissolved in a mixture of Ac_2O (500 μl) and dry pyridine (700 μl) and the soln left at 30° for 18 hr. The residue obtained by evaporation of the reagents under red pres. was dissolved in MeOH which was then evaporated. The residue was purified on TLC (silica gel, eluent C) yielding chromatographically pure **5** (1.5 mg): $[\alpha]_D^{25}$ $-19.0°$ ($CHCl_3$, *c* 0.08), UV $\lambda_{\text{max}}^{\text{EtOH}}$ nm: 268 ($\varepsilon = 6.3 \times 10^3$); IR $\nu_{\text{max}}^{\text{CHCl}_3}$ cm^{-1}: 1750 (C=O); ^{1}H NMR: see Table 1; MS (EI), 70 eV, *m/z* (rel. int.): 533 $[M]^+$ (1.2), 518 (0.2), 474 (60 0), 432 (1.2), 259 triacetylriboside (8 5), 216 (100.0), 200 (20.0), 199 (8.5), 157 (7 3), 139 (52.0), 97 (52 0).

Hydrolysis of **3**. Compound **3** (0.3 mg) was hydrolysed by heating at 95° for 60 min in 0.5 M HCl (500 μl). TLC analysis (silica gel and cellulose, eluent A and reverse phase eluent D) showed the formation of β-D-ribose. This was confirmed by co-chromatography with an authentic sample

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*Nomenclature: zeatin (Z): 6-(4-hydroxy-3-methylbut-*trans*-2-enylamino)purine; isopentenyladenine (iP): N^6-(Δ^2-isopentenyl)adenine; isopentenyladenosine (iPA): 9-β-D-ribofuranosyl-iP.

†National Collection Plant Pathogenic Bacteria, Harpenden, U.K.

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