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PUTAMINOXINS B AND C FROM *PHOMA PUTAMINUM*

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Key Word Index—*Erigeron annuus*; Compositae; *Phoma putaminum*; phytotoxins; herbicides; macrolides; nonenolides; polyketides; hexaketides; putaminoxins B and C.

Abstract—A phytotoxic macrolide and a hexaketide, putaminoxins B and C, respectively, were isolated together with putaminoxin, the main phytotoxin, from liquid culture filtrates of *Phoma putaminum*, the causal agent of leaf necrosis of *Erigeron annuus*, a common weed of fields and pastures. Putaminoxins B and C were characterized, using spectroscopic methods, as a new disubstituted nonenolide and a new disubstituted cyclononendione, respectively. Assayed on leaves of weedy and cultivated host plants, putaminoxins C showed toxic effects similar to those produced by putaminoxin. Copyright © 1997 Elsevier Science Ltd

INTRODUCTION

Recent studies on the toxic metabolites produced by *Phoma putaminum*, a fungal pathogen responsible for the necrotic leaf disease of *Erigeron annuus* (L.) Pers., commonly known as annual fleabane, an indigenous weed from North America widely found in fields and pastures all over Europe, including Italy, led to the isolation of the main phytotoxin, putaminoxin (**1**) [1], a new disubstituted nonenolide. Putaminoxin had no zootoxicity and, when assayed on leaves of weeds and cultivated plants, showed a highly interesting semiselective phytotoxic activity against the host plant [1]. Considering that phytotoxins could be used directly as herbicides or as analogues for the development of selective non-persistent herbicides [2] and that the organic culture extract of the fungus showed high phytotoxic activity, further investigation of this extract was carried out to identify any other associated toxins. Two metabolites structurally related to putaminoxin were isolated. This paper describes the isolation and the chemical and biological characterization of these two new putaminoxins (B and C).

RESULTS AND DISCUSSION

The crude oily residue, obtained by ethyl acetate extraction of the culture filtrates of *P. putaminum*, was fractioned using a combination of column chromatography and TLC steps (see Experimental) to yield putaminoxin (**1**) and two other metabolites with a chromatographic behaviour very similar to that of **1**. The two metabolites, obtained as homogeneous compounds withstanding crystallization, were named putaminoxin B (**2**, 0.25 mg l^{-1}) and putaminoxin C (**3**, 0.43 mg l^{-1}) on the basis of their structural relationship to **1**, as evidenced by a preliminary spectroscopic investigation (see below).

When assayed on punctured and detached leaves of several weeds and cultivated plants at 8 μg/droplet, after 2 days putaminoxin C caused the appearance of necrotic spots only on *Mercurialis annua* (annual dog's mercury) and on *Cynara cardunculus* (globe artichoke) and chlorosis on *Lycopersicon esculentum* (tomato), while no toxic effects were observed on *Vicia faba* (faba bean), *Sorghum bicolor* (sorghum) or *Cucumis sativus* (cucumber), indicating a semiselectivity comparable to that of putaminoxin. Assayed at lower concentration, only the first dilution (4 μg/droplet) still caused toxic effects. On the other hand, no phytotoxicity was shown by putaminoxin B. Assayed on *Bacillus megaterium* and *Pseudomonas* sp. at up to 40 μg/disk, only putaminoxin C caused a clear inhibition halo of the growth of *B. megaterium*; no such inhibition was shown by either putaminoxin or putaminoxin B. No zootoxic or antifungal activity was observed for either compound (**2** and **3**) when assayed up to 5×10^{-5} M on *Artemia salina* (brine shrimps) and up to 50 μg/disk on *Geotrichum candidum*.

Putaminoxin B showed only an end-UV absorption, while its IR spectrum had bands characteristic of hyd-

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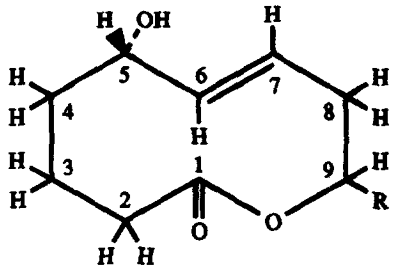
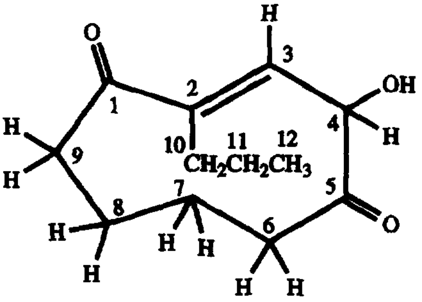


10 11 12
1 R=$CH_2CH_2CH_3$

10 11 12 13 14
2 R=$CH_2CH_2CH_2CH_2CH_3$



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roxy, carbonyl and olefinic groups [3]. The structural relationship of this metabolite with **1** was deduced from the examination of its ^1H and ^{13}C NMR spectra. In fact, the ^1H NMR spectrum (Table 1) of **2**, as compared to that of **1**, showed more complex systems in the region of the aliphatic protons δ 2.41–1.20 as the only substantial difference [4]. Whereas, the system attributable to the partial structure containing the secondary hydroxyl group at C-5 with an (*S*) configuration and the *trans*-disubstituted olefinic group between C-6 and C-7 together with that of a terminal aliphatic side chain, probably attached at C-9, were present also in **2**. Therefore, a structure of a disubstituted nonenolide with a longer side chain was hypothesized for putaminoxin B. These structural features were also observed in the ^{13}C NMR spectrum (Table 1), which differed from that of **1** only in the presence of the signals of two other aliphatic methylene groups [5], which probably belong to a pentyl residue attached at C-9, while in **1** this side chain is a propyl group.

Table 1. ^1H and ^{13}C NMR data for putaminoxins B (**2**) and C (**3**)

2* ($CDCl_3$)					3 (C_6D_6)†				
C	δ	m‡	^1H δ	m	C	δ	m‡	^1H δ	m
1	172.5	*s*	–	–	1	197.2	*s*	–	–
2	32.6	*t*	2.35	*m*	2	138.0	*s*	–	–
			1.85	*m*					
3	23.2	*t*	2.28	*m*	3	148.7	*d*	6.53	*t*
			1.90–1.20	*m*					
4	34.3	*t*	1.82	*m*	4	74.8	*d*	4.99	*d*
			1.50	*m*					
5	71.5	*d*	4.11	*ddd*	5	208.8	*s*	–	–
6	135.5	*d*	5.40	*ddd*	6	38.1	*t*	2.07 (2H)	*m*
7	130.0	*d*	5.50	*ddd*	7	22.5	*t*	1.34 (2H)	*m*
8	39.0	*t*	2.41	*m*	8	26.0	*t*	1.63 (2H)	*m*
			2.18	*m*					
9	73.1	*d*	5.10	*m*	9	37.5	*t*	2.34 (2H)	*t*
10	37.2	*t*	1.90–1.20 (2H)	*m*	10	25.6	*t*	1.57 (2H)	*td*
11	24.0	*t*	1.70 (2H)	*m*	11	22.5	*t*	1.22 (2H)	*tq*
12	30.0	*t*	1.57 (2H)	*m*	12	13.8	*q*	0.84 (3H)	*t*
13	18.8	*t*	1.36 (2H)	*m*	HO		–	4.29	*d*
14	13.9	*q*	0.95 (3H)	*t*					

The chemical shifts are δ values (ppm) from TMS.

J (Hz) **2**: 4,5 = 9.9; 4′,5 = 4.0; 5,6 = 9.2; 6,7 = 15.0; 6,8 = 1.5; 7,8 = 3.3; 7,8′ = 10.7; 13,14 = 7.3. **3**: 3,10 = 4.0; 4,OH = 5.0; 8,9 = 7.0; 10,11 = 11,12 = 7.2.

*Assigned in agreement with the evidence obtained from a COSY experiment and the value recorded for putaminoxin [1].

†COSY and HMQC NMR experiments delineated the correlations of all protons and the corresponding carbon atoms.

‡Multiplicities determined by DEPT spectra.

Considering the already known chemical shifts of the four methylene groups of the macrocyclic ring (H_2C-2, H_2C-3, H_2C-4 and H_2C-8) of **1** and the theoretical chemical shifts calculated for a suitable aliphatic side chain [5], the chemical shifts were assigned to all carbon atoms confirming the presence of the pentyl residue at C-9. In fact, by reference to the signals reported for the same moiety in **1**, the signals at δ 30.0 and 24.0 were assigned to H_2C-12 and H_2C-11, respectively, while those at δ 37.2, 18.8 and 13.9 were attributed to H_2C-10, H_2C-13 and Me-14, respectively.

The presence of a pentyl side chain was also supported by the correlations observed in the COSY-45 (correlation spectroscopy) [6] spectrum of **2** and by the data from its electron impact mass spectrum. In fact, the latter showed $[M]^+$ at *m/z* 240, and fragmentation peaks generated from mechanisms similar to those observed in **1** [1], typical of macrolides [4, 7]. In fact, the molecular ion, probably by consecutive losses of C_2H_4, CO_2 and C_3H_7, generated ions at *m/z* 212, 168 and 125 (base peak), respectively. The ion at *m/z* 168, by the loss of C_4H_9 or C_5H_{11}, yielded the ions at *m/z* 111 and 97, respectively. Alternatively, loss of water from $[M]^+$ followed by that of Me or C_2H_4 residues produced the ions at *m/z* 222, 207 or 194, respectively. Based on these results putaminoxin B may be formulated as (5*S*)-5-hydroxy-9-pentyl-6-nonen-9-olide (**2**).

Putaminoxin C (**3**) had a molecular weight of 210, as deduced from its electron impact mass spectrum, and therefore should differ from **1** by two hydrogen atoms, with the molecular formula $C_{12}H_{18}O_3$. The IR spectrum of **3** showed bands typical of hydroxyl and saturated carbonyl groups and those of at least one α,β-unsaturated carbonyl group. The presence of the latter was confirmed by the UV absorption maximum at 229 nm, which is typical of an enone system with some steric hindrance to coplanarity [8].

These structural features and their correlation with the structure of **1** were confirmed by examination of the ^1H NMR spectrum of **3** (Table 1). In fact, this spectrum contained signals of a propyl residue attached at the α-carbon of the α,β-unsaturated carbonyl group. The olefinic proton (H-3) resonating as a triplet at δ 6.53 was coupled ($J_{3,10} = 4.0$ Hz) with the attaching methylene group (H_2C-10) of the side chain, which appeared as a triple doublet at δ 1.57. The large value of this allylic coupling constant indicates *E* stereochemistry of the trisubstituted double bond located between C-2 and C-3, in agreement with literature data [9]. A triplet and a multiplet typical of methylene groups in the α-position with respect to a carbonyl group [4] were observed at δ 2.34 and 2.07 (H_2C-9 and H_2C-6, respectively). Moreover, other systems, probably due to two other alicyclic methylene groups, were present, together with those of the propyl side chain at δ 1.57–0.84. Finally, the doublet of the proton (H-4) of a secondary alcohol resonating at δ 4.99 was coupled with the geminal hydroxyl proton, which appeared as a doublet at δ 4.29. As expected, when the spectrum was recorded in $CDCl_3$ with addition of D_2O, the latter dissappeared and the H-4 signal was replaced by a sharp singlet at δ 4.91. The hydroxyl group might be located at the γ-carbon (C-4) of the α,β-unsaturated system and, considering the multiplicity and chemical shift value of H-4, in the α-position with respect to another carboxyl group. This latter resonates as a singlet at δ 208.8, being deshielded by the intramolecular hydrogen bond that is probably formed with HOC-4 [5]. The ^{13}C NMR data (Table 1) confirmed these structural features. In fact, the signals of the α,β-unsaturated carbonyl group appeared at the typical chemical shift values [5] of δ 197.2 (C-1), 138.0 (C-2) and 148.7 (C-3) for the carbonyl group and the two carbon atoms of the trisubstituted double bond. The C-4 resonated as doublet at δ 74.8, while the signals of the two methylene groups located in the α-position relative to the two carbonyl groups were observed at δ 38.1 and 37.5 (C-6 and C-9, respectively) [5]. One- and two-dimensional experiments, using COSY-45 [6] and heteronuclear multiple-quantum correlation (HMQC) techniques [10] confirmed these assignments and allowed assignment of the chemical shifts associated with the protons and the corresponding carbon atoms of the propyl side chain and the remaining two methylene groups, as reported in Table 1.

Considering these findings, the structure of a disubstituted cyclononendione might be suggested for putaminoxin C (**3**). This structure correlates with that of **1**, except that it lacks the lactone group, and the hydroxyl and propyl groups are differently located in the macrocyclic ring. This structure is supported by the electron impact mass spectrum, which shows $[M]^+$ at *m/z* 210 and fragmentation peaks typical of hydroxylated alicyclic ketones also containing a propyl side chain [4]. In fact, loss of C_2H_5 or C_3H_7 from $[M]^+$ generated ions at *m/z* 181 and 167, respectively. The alternative loss of H_2O from the latter produced the ion at *m/z* 149. Finally, the ion at *m/z* 181 by the loss of C_3H_7 or C_2H_4 followed by CO yielded the ions at *m/z* 138 and 125 (base peak), respectively. Therefore, putaminoxin C may be formulated as 4-hydroxy-2-propyl-2-cyclononene-1,5-dione (**3**).

In conclusion, we have satisfactorily demonstrated the structure of a new disubstituted nonenolide and a new disubstituted cyclononendione for putaminoxins B and C, respectively. Putaminoxin B, as already considered for putaminoxin (**1**) [1], is a macrolide, a well-known family of naturally occurring compounds with interesting biological activity, whereas putaminoxin C seems to have a polyketide origin [11–13]. Macrolides and polyketides are both fungal metabolites [1, 11, 12]. Considering that these two new metabolites showed modified biological activity with respect to those of putaminoxin, and also considering the interesting phytotoxicity of putaminoxin, it seems to be desirable to obtain some derivatives that could have a different selectivity or an increased toxicity with respect to the natural compound.

EXPERIMENTAL

General. IR and UV: neat and MeCN, respectively; ^{1}H and ^{13}C NMR: at 400 and/or 270 MHz and 100 and/or 67.92 MHz, respectively, using the same solvent as internal standard as reported in Table 1. Carbon multiplicities were determined from distortionless enhancement by polarization transfer (DEPT) spectra [5]. DEPT, COSY-45 and HMQC experiments were performed using Bruker standard microprograms; EIMS: 70 eV. Analytical and preparative TLC: silica gel (Merck, Kieselgel 60 F_{254}, 0.25 and 0.50 mm, respectively) or reverse phase (Whatman, KC-18 F_{254}, 0.20 mm) plates; spots were visualized by exposure to UV radiation and/or by spraying first with 10% H_2SO_4 in MeOH and then with 5% phosphomolybdic acid in MeOH, followed by heating at 110° for 10 min; CC: silica gel (Merck, Kieselgel 60, 0.063–0.20 mm); solvent systems: (A) $CHCl_3$/iso-PrOH (19:1); (B) EtOAc/n-hexane (1.5:1); (C) $CHCl_3$/iso-PrOH (9:1); (D) EtOH/H_2O (1.5:1); (E) $CHCl_3$/iso-PrOH (32.3:1).

Production, extraction and purification of putaminoxins. The isolation of *P. putaminum* from diseased leaves of *E. annuus*, its growth on M-1-D medium and the extraction procedure of its toxic metabolites were as described in [1]. The organic (EtOAc) extract (3.57 g) obtained from the culture filtrates (12 l) was fractioned by CC column with eluent A to yield 9 groups of homogeneous frs. The pooled fraction groups between 1–6 and 8 showed phytotoxic activity. The residue (91.1 mg) left from group 4, containing the main metabolite was further purified by a combination of successive prep. TLC steps on silica gel and reverse phase yielding putaminoxin (**1**) as a homogeneous oily compound (24 mg) withstanding crystallization. A further amount of **1** (5.2 mg; total 2.4 mg l^{-1}) was obtained from fr. group 5 of the initial column. The residue (136 mg) left from fr. group 3 of the original column, containing metabolites with higher R_f values (TLC on silica gel, eluent A) than that (0.41) of putaminoxin were purified by prep. TLC (silica gel, eluent B) producing two frs: A (R_f 0.52, 33.6 mg) and B (R_f 0.38, 16.4 mg). The residue of band A was further purified by two successive prep. TLC on silica gel (eluent A) and reverse phase (eluent D) to yield putaminoxin B (**2**, 3.0 mg, 0.25 mg l^{-1}) as homogeneous compound withstanding crystallization. Fraction B was further purified by reverse phase prep. TLC (eluent D) to give putaminoxin C (**3**, 5.2 mg, 0.43 mg l^{-1}) also as homogenous oil resisting crystallization.

Putaminoxin B (**2**). UV λ_{max} nm (log ε) < 220; IR ν_{max} cm^{-1}: 3288 (OH), 1725 (C=O), 1665 (C=C), 1153 (O–CO), 1054 (O–CO); ^{1}H and ^{13}C NMR spectra: Table 1; EIMS, *m/z* (rel. int.): 240 $[M]^+$ (9), 223 $[M-OH]^+$ (12), 222 $[M-H_2O]^+$ (11), 212 $[M-C_2H_4]^+$ (4), 207 $[M-H_2O-Me]^+$ (9), 197 $[M-C_2H_4-Me]^+$ (12), 194 $[M-H_2O-C_2H_4]^+$ (8), 168 $[M-C_2H_4-CO_2]^+$ (82), 140 $[M-C_2H_4-CO_2-CO]^+$ (71), 125 $[M-C_2H_4-CO_2-C_3H_7]^+$ (100), 111 $[M-C_2H_4-CO_2-C_4H_9]^+$ (43), 97 $[M-C_2H_4-CO_2-C_5H_{11}]^+$ (70).

Putaminoxin C (**3**). UV λ_{max} nm (log ε) 229 (3.49); IR ν_{max} cm^{-1}: 3444 (OH), 1713 (C=O), 1673 (C=O), 1600 (C=C). The ^{1}H and ^{13}C NMR spectra: Table 1; EIMS, *m/z* (rel. int.): 210 $[M]^+$ (1), 193 $[M-OH]^+$ (1.5), 181 $[M-C_2H_5]^+$ (1), 167 $[M-C_3H_7]^+$ (3), 149 $[M-C_3H_7-H_2O]^+$ (16), 138 $[M-C_2H_5-C_3H_7]^+$ (11), 126 $[M-C_2H_5-CH_2{=}CH-CO]^+$ (68), 125 $[M-C_2H_5-C_2H_4-CO]^+$ (100), 97 (36), 57 (66).

Biological methods. Each sample was dissolved in a small amount of MeOH and brought to the required concn with distilled H_2O or sea water soln (final concn of MeOH 4% and 1%, respectively). The phytotoxic activity on weedy and cultivated species, as well as the zootoxic and antimicrobial activities were tested following the method reported in [1].

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