Pergamon

Phytochemistry, Vol. 48, No. 6, pp. 941–945, 1998

Printed in Great Britain
0031-9422/98 $19.00 + 0.00

PII: S0031-9422(97)00879-0

PUTAMINOXINS D AND E FROM *PHOMA PUTAMINUM*

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(*Received in revised form* 16 *September* 1997)

Key Word Index—*Erigeron annuus*; Compositae; *Phoma putaminum*; phytotoxins; herbicides; 10-macrolides; nonen- and nonan-olides; putaminoxins D and E; 5-*O*-formylputaminoxin.

Abstract—Two 10-macrolides, named putaminoxins D and E, were isolated together with other recently identified toxic putaminoxins from culture filtrates of *Phoma putaminum*, the causal agent of leaf necrosis of *Erigeron annuus*, a common weed found in fields and pastures. Putaminoxins D and E were characterized by spectroscopic and chemical methods as a new disubstituted nonen- and nonanolide, respectively. Moreover, 5-*O*-formylputaminoxin was isolated as an artefact from the culture filtrates acidified with formic acid. When they were assayed on leaves of host and non-host plants, putaminoxins D and E and 5-*O*-formylputaminoxin showed no toxicity, unlike other putaminoxins.

INTRODUCTION

Phoma putaminum (Speg.) is a fungal pathogen responsible for a necrotic leaf disease of *Erigeron annuus* (L.) Pers. which is commonly known as annual fleabane. *E. annuus* is an indigenous weed from North America and is widely found in fields and pastures all over Europe, including Italy. Three phytotoxic metabolites were isolated from the EtOAc extract of the culture filtrates and characterized as two new disubstituted nonenolides, namely putaminoxin (**1**) [1] and putaminoxins B (**2**) [2], and a new disubstituted cyclononendione, named putaminoxin C [2].

The organic cultural extract contained at least two other metabolites present at very low concentrations and their chromatographic behaviour and structures were related to both putaminoxin and putaminoxin B. This paper describes the isolation and chemical and biological characterization of these two new disubstituted 10-macrolides, named putaminoxins D and E, as well as those of the 5-*o*-formylputaminoxin, which proved to be an artefact of the extraction procedure.

RESULTS AND DISCUSSION

The crude oily residue, obtained by extraction (EtOAc) of the culture filtrates of *P. putaminum*, was fractionated using a combination of CC and TLC steps (see Experimental) to yield putaminoxin (**1**) [1], and putaminoxins B (**2**) and C [2] as well as two other metabolites whose chromatographic behaviour was very similar to that of **1**. The two metabolites, which were obtained as homogeneous compounds withstanding crystallization, were named putaminoxin D (**3**, 0.13 mg l^{-1}) and putaminoxin E (**6**, 0.13 mg l^{-1}) on the basis of their structural relation with **1** and **2** evidenced by a spectroscopic investigation as shown below. Putaminoxin D and E showed only an end-UV absorption. The structural relationship of these two metabolites with both **1** and **2** was deduced from the examination of their ^1H and ^{13}C NMR spectra.

In fact, the only substantial difference in the ^1H NMR spectrum of **3**, compared to that of **1**, showed more complex systems in the region of the aliphatic protons δ 2.40–1.30 as already observed in **2** [3]. Furthermore, the two olefinic protons (H-6 and H-7) showed a complex overlapping signal at δ 5.47 while in the ^{13}C NMR spectrum the corresponding carbons appeared as a doublet at the expected δ values of 134.4 (C-6) and 133.0 (C-7) [4]. In the latter spectrum the signals of eight methylenes were also observed between δ 39.8 and 16.5 [4]. These findings together with a molecular weight of 240, as deduced from its EI-MS spectrum, suggested a structure which was very close to **2** for this phytotoxin, except it had a different stereochemistry of the double bond [C(6)=C(7)] of the macrocyclic ring. Such a hypothesis was ruled out by the examination of the ^1H NMR spectrum of the 5-*O*-acetyl derivative of putaminoxin D (**4**, $[M]^+ = 282$ *m/z* by EI-MS). In fact, this differed from that of **3**, because of the expected downfield shift ($\Delta\delta$ 1.05) of the doublet of double doublets (J = 10.2 Hz,

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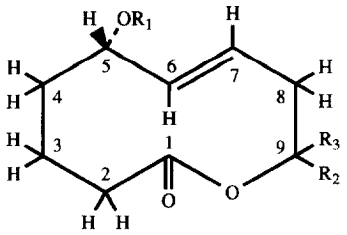

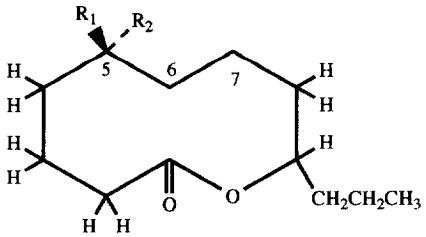

1 R_1=H, R_2=$CH_2CH_2CH_3$, R_3=H

2 R_1=H, R_2=$CH_2CH_2CH_2CH_2CH_3$, R_3=H

3 R_1=H, R_2=H, R_3=$CH_2CH_2CH_2CH_2CH_3$

4 R_1=Ac, R_2=H, R_3=$CH_2CH_2CH_2CH_2CH_3$

5 R_1=HCO, R_2=$CH_2CH_2CH_3$, R_3=H

6 R_1=OH, R_2=H

7 R_1=H, R_2=OH

9.8 Hz and 4.1 Hz) of H-5 at δ 5.14 as well as the surprising appearance of the olefinic protons in the form of two well resolved systems. The latter (H-7 and H-6) resonated as a doublet of double doublets (J = 15.8 Hz, J = 10.5 Hz, and J = 3.7 Hz) and as a double doublet (J = 15.8 Hz and 9.8 Hz) at δ 5.61 and 5.44, respectively. The values of the coupling constants measured for these systems made it possible to assign a *trans*-stereochemistry to the double bond of the macrocyclic ring located between C-6 and C-7 [5]. Moreover, considering that also the coupling constants between H-5 and H-6 and both protons of H_2C-4, in respect to **1** and **2**, appeared unchanged, it is possible to attribute the same relative configuration at C-5 as indicated for this secondary carbon in both putaminoxin and putaminoxin B [1, 2].

On the basis of these results the epimer structure at C-9 of **2** was suggested for putaminoxin D. As expected, the latter proved to be different from **2** when co-chromatographed in three different systems (silica gel, eluents A and B, reverse phase, eluent D).

The structure assigned to **3** was also proven by the fragmentation mechanisms observed in its EI-MS spectrum, which were very similar to those already observed in **2** [2]. In fact, when the molecular ion successively lost C_2H_4 and CO_2, it generated the ions at *m/z* 212 and 168 [3, 6]. The latter corresponding to the intermediate 3-hydroxy-5-pentylcyclohexene ion, which had been found already [2], generated the ions at *m/z* 125 (base peak) and 97 by successive losses of C_3H_7 and C_5H_{11}, respectively. Moreover, by alternative fragmentation pathways, the molecular ion lost H_2O or C_2H_6 followed by CO and yielded the ions at *m/z* 222 and 210 and 182, respectively [3, 6]. Similar fragmentation mechanisms were observed in the EI-MS spectrum of the 5-*O*-acetyl derivative **4**. When the molecular ion at *m/z* 282 successively lost CH_2CO, C_2H_4 and CO_2 it produced the above mentioned intermediate ion at *m/z* 168 [3, 6], and when this alternatively lost the residue C_4H_9 or C_5H_{11} it produced the ions at *m/z* 111 (base peak) and 97, respectively. As expected, when the molecular ion alternatively lost Me followed by HOAc it generated the ions at *m/z* 267 and 207, respectively [3]. Moreover, the FAB-MS spectrum of **3** showed the pseudomolecular ion $[M]^+$ at *m/z* 241 and the significant ion at *m/z* 225 generated from this latter by loss of Me. Thus putaminoxin D may be formulated as (5*S*)-5-hydroxy-9-pentyl-6-nonen-9-olide.

Putaminoxin E (**6**) showed a molecular weight of 214, as deduced from its EI-MS spectrum, and this differs from the putaminoxin because of two more hydrogen atoms as was also revealed from the examination of its ^{1}H NMR spectrum. The latter basically differed from that of **1** due to the absence of olefinic protons, because of the major complexity of the multiplet of H-5 resonating at δ 4.13 and further complex systems in the region of the aliphatic systems between δ 2.18–1.10 [3]. As expected, its ^{13}C NMR spectrum differed from that of **1** for a significant upfield shift ($\Delta\delta$ 5.8 and 10.5, respectively) of C-5 and C-8 at δ 68.2 and 29.8, and in the presence of the signals of two more methylene groups at δ 29.8 and 21.2 attributed to C-6 and C-7, respectively. These differences were similar to those already observed when the ^{1}H and ^{13}C NMR spectra of 5,6-dihydroputaminoxin (**7**), obtained by catalytic hydrogenation of putaminoxin [1], were compared to those of **1**. Thus the structure of a 5,6-dihydroputaminoxin was suggested for **6**.

The structure of putaminoxin E was consistent with the fragmentation peaks observed in its EI-MS spectrum. In fact, when the molecular ion (*m/z* 214) successively lost C_2H_4 and CO_2 it generated the ion at *m/z* 142 [3, 6]. When the latter, corresponding to the

probable intermediate 3-propylcyclohexanol ion, alternatively lost Me followed by H_2O or C_3H_7 it yielded the ions at *m/z* 127 and 109 and 99, respectively. Lastly, by an alternative fragmentation mechanism, when the molecular ion successively lost OH and Me it produced the ions at *m/z* 197 (base peak) and 182, respectively [3].

The above findings, as well as the different chromatographic behaviour of **6** when co-chromatographed with 6,7-dihydroputaminoxin in the three above mentioned systems, suggested the structure of a 5 or a 9-epimer of **7** for this putaminoxin. However, a further comparison of the ^1H NMR spectrum of **6** with that of **7** showed a very similar chemical shift value in the H-9 multiplet (δ 4.98 and 4.92, respectively) while that of H-5 proved to be very different (δ 4.13 and 3.55, respectively). On the basis of these results putaminoxin E may be formulated as 5-epi-6,7-dihydroputaminoxin.

Furthermore, a compound (**5**, 0.78 mg l^{-1}) was isolated from the lesser polar fraction of the initial column, and showed a structure which was very similar to that of putaminoxin (**1**). In fact, its ^1H NMR only differed from that of **1** in the downfield shift ($\Delta\delta$ 1.21) of the doublet of double doublets (J = 9.7 Hz, J = 9.6 Hz and J = 3.2 Hz) of H-5 at δ 5.19 as a consequence of the esterification of the geminal hydroxy with a formyl group appearing as a singlet at a typical δ value of 7.98 [3]. As expected, the only substantial difference in the ^{13}C NMR spectrum, compared to that of **1**, was the presence of the doublet of the formyl group resonating at δ 160.1 [3, 4]. Consequently, in agreement with the molecular weight of 240, compound **5** proved to be the 5-*O*-formylputaminoxin. This structure was supported by the fragmentation ions observed in its EI-MS spectrum. In fact, when the molecular ion successively lost CO, C_2H_4 and CO_2 it generated the ions at *m/z* 212 and 140 [3, 6]. The latter is the base peak corresponding to the hypothesized 3-hydroxy-5-propylcyclohexene ion already found in the spectrum of putaminoxin (**1**) [1]. When the ion at *m/z* 140 successively lost Me and H_2O it produced the ions at *m/z* 125 and 107. Furthermore, when the molecular ion alternatively lost CO and H_2O or HCOO it yielded the ions at *m/z* and at 194 and 195, respectively [3, 6].

When assayed on tomato and annual fleabane leaves at a concentration of 4 10^{-3} M putaminoxins D and E, as well as 5-*O*-formylputaminoxin, showed no phytotoxicity, indicating that the presence of the unalterated alkyl side chain at C-9 and that both the double bond and the hydroxy group at C-5 of the macrocyclic ring are structural features which are important for the activity. These results agree with the lack of phytotoxicity previously showed by putaminoxin B [2] which as **3** showed a pentyl side chain at C-9 although it had an opposite configuration. In addition, these metabolites proved to be inactive on the brine shrimps (*Artemia salina*) assay (tested at 4 10^{-4} M) and on *Geotrichum candidum* (at 20 μg disk) as already observed in tests with both putaminoxin and putaminoxin B [1, 2]. Putaminoxin C, which is a disubstituted cyclononenedione and therefore quite different from putaminoxins, showed toxicity on plants and bacteria [2].

5-*O*-formylputaminoxin proved to be an artefact from the extraction process because it was the main component of the crude extract when the extraction was performed after acidification with HCOOH, but it was absent when the culture filtrates were acidified with HCl and extracted under the same conditions.

EXPERIMENTAL

General

IR and UV: neat and MeCN, respectively; ^1H and ^{13}C NMR: in $CDCl_3$ at 400 and/or 270 MHz and 100 and/or 67.92 MHz, respectively, using the same solvent as internal standard. Carbon multiplicities were determined by DEPT (Distortionless Enhancement by Polarization Transfer) spectra [4]. DEPT experiments were performed using a Bruker standard microprogram; EI-MS: 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; the 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 recently described in detail [1]. The organic (EtOAc) extract (3.57 g) obtained from the culture filtrates (12 l) was fractionated by CC column with eluent A to yield 9 groups of homogeneous frs. The pooled fr. 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 R_f values higher (TLC on silica gel, eluent A) than that (0.41) of putaminoxin, was a very complex mixture. It was purified by prep. TLC (silica gel, eluent B) and produced five frs. The residue (33.6 mg) of the fr. with R_f 0.52 was further fractionated on the same system

but this time using the eluent system C. The residue (7.2 mg) obtained by the elution of the band with R_f 0.41 proved to contain a mixture of three metabolites when chromatographed on reversed phase (eluent D). Therefore, when it was purified using the same system it yielded putaminoxins B, D and E (**2**, **3** and **6**; 3.0, 1.5 and 1.5 mg, 0.25, 0.13 and 0.13 mg l^{-1}, respectively) in the form of a homogeneous oil (R_f 0.38, 0.52 and 0.45, respectively) resisting crystallization. The main component of the residue (29 mg) of first fr. of the initial column was the lesser polar compound (**5**) of the organic extract as shown by TLC on silica gel (R_f 0.49, eluent E). When this residue was purified by two successive prep. TLC steps on silica gel (eluent E and A, respectively) it yielded compound **5**, identified as the 5-*O*-formylputaminoxin, as a homogeneous oil (9.3 mg, 0.78 mg l^{-1}) withstanding crystallization.

Putaminoxin D (**3**). Putaminoxin D (**3**) had: UV λ_{max} nm (log ε) < 220; ^{1}H NMR, δ: 5.47 (2H, *m*, H-6 and H-7), 5.05 (1H, *m*, H-9), 4.09 (1H, *ddd*, J = 11.3 Hz, J = 8.7 Hz and J = 4.0 Hz, H-5), 2.40–1.30 (16H, *m*, eight CH_2), 0.91 (3H, *t*, J = 7.3 Hz, Me-14); ^{13}C NMR, δ: 171.0 (*s*, C-1), 134.4 (*d*, C-6), 133.0 (*d*, C-7), 71.0 (*d*, C-9), 69.2 (*d*, C-5), 39.8 (*t*, C-8), 36.0 (*t*, C-10), 33.0 (*t*, C-4), 31.9 (*t*, C-2), 27.6 (*t*, C-12), 27.6 (*t*, C-11), 24.6 (*t*, C-3), 16.5 (*t*, C-13), 12.0 (*q*, C-14); EI-MS, *m/z* (rel. int.): 240 $[M]^+$ (1), 223 $[M-OH]^+$ (3), 222 $[M-H_2O]^+$ (12), 212 $[M-C_2H_4]^+$ (1), 210 $[M-C_2H_6]^+$ (20), 182 $[M-C_2H_6-CO]^+$ (7), 167 $[M-C_2H_6-CO-Me]^+$ (8), 168 $[M-C_2H_4-CO_2]^+$ (2), 125 $[M-C_2H_4-CO_2-C_3H_7]^+$ (100), 97 $[M-C_2H_4-CO_2-C_5H_{11}]^+$ (20). FAB (+), *m/z* (rel. int.): 241 $[MH]^+$ (100), 225 $[M-Me]^+$ (98).

5-O-*Acetylputaminoxin D* (**4**). Putaminoxin D (**3**, 0.66 mg) was acetylated with pyridine (30 μl) and Ac_2O (30 μl) at room temp. overnight. The oily residue left by the reaction work-up was purified by preparative TLC (silica gel, eluent A) to give **4** as a homogeneous compound (0.50 mg). UV λ_{max} nm (log ε) < 220; ^{1}H differed from that of **3** in the following systems, δ: 5.61 (1H, *ddd*, J = 15.8 Hz, J = 10.5 Hz and J = 3.7 Hz, H-7), 5.44 (1H, *dd*, J = 15.8 Hz and 9.8 Hz, H-6), 5.14 (1H, *ddd*, J = 10.2 Hz, 9.8 Hz and 4.1 Hz, H-5), 2.02 (3H, *s*, MeCO); EI-MS, *m/z* (rel. int.): 282 $[M]^+$ (5), 281 $[M-H]^+$ (15), 267 $[M-Me]^+$ (2.5), 223 $[M-AcO]^+$ (1), 207 $[M-Me-AcOH]^+$ (60), 168 $[M-CH_2CO-C_2H_4-CO_2]^+$ (5), 167 $[M-CH_2CO-C_2H_5-CO_2]^+$ (31), 150 $[M-H-AcOH-C_5H_{11}]^+$ (12), 149 $[M-CH_2CO-C_2H_5-CO_2-H_2O]^+$ (100), 111 $[M-CH_2CO-C_2H_4-CO_2-C_4H_9]^+$ (22), 97 $[M-CH_2CO-C_2H_4-CO_2-C_5H_{11}]^+$ (39).

5-O-*Formylputaminoxin* (**5**). 5-*O*-formylputaminoxin had: UV λ_{max} nm (log ε) < 220; ^{1}H NMR δ: 7.98 (1H, HCO), 5.72 (1H, *ddd*, J = 15.0 Hz, J = 10.6 Hz and J = 4.9 Hz, H-7) 5.29 (1H, *dd*, J = 15.0 Hz and J = 9.6 Hz, H-6), 5.19 (1H, *ddd*, J = 9.7 Hz, J = 9.6 Hz and J = 3.2 Hz, H-5), 5.05 (1H, *m*, H-9), 2.42 (1H, *m*, H-2), 2.36 (1H, *m*, H-8), 2.06–1.26 (10H, *m*, H-2′, H-8′ and four CH_2), 0.93 (3H, *t*, Me-12) ^{13}C NMR, δ: 175.4 (*s*, C-1), 160.1 (*d*, HCO), 75.8 (*d*, C-9), 75.0 (*d*, C-5), 40.3 (*t*, C-8), 36.4 (*t*, C-10), 35.6 (*t*, C-2), 35.5 (*t*, C-4), 22.1 (*t*, C-3), 19.1 (*t*, C-11), 13.8 (*q*, C-12); EI-MS, *m/z* (rel. int.): 240 $[M]^+$ (1), 212 $[M-CO]^+$ (4), 211 $[M-HCO]^+$ (3), 195 $[M-HCOO]^-$ (9), 194 $[M-CO-H_2O]^+$ (49), 168 $[M-C_2H_4-CO_2]^+$ (97), 140 $[M-CO-C_2H_4-CO_2]^-$ (100), 125 $[M-CO-C_2H_4-CO_2-Me]^+$ (97), 107 $[M-CO-C_2H_4-CO_2-Me-H_2O]^+$ (66).

Putaminoxin E (**6**). Putaminoxin E (**6**) had: UV λ_{max} nm (log ε) < 220; ^{1}H NMR, δ: 4.98 (1H, *m*, H-9), 4.13 (1H, *m*, H-5), 2.45 (1H, *ddd*, J = 14.8 Hz, J = 8.7 Hz and J = 3.7 Hz, H-2), 2.23 (1H, *ddd*, J = 14.8 Hz, J = 11.1 Hz and J = 3.7 Hz, H-2′), 2.18–1.10 (14H, *m*, seven CH_2), 0.94 (3H, *t*, J = 7.3 Hz, Me-12); ^{13}C NMR, δ: 173.5 (*s*, C-1), 76.7 (*d*, C-9), 68.2 (*d*, C-5), 35.5 (*t*, C-10), 35.0 (*t*, C-2), 31.8 (*t*, C-4), 29.8 (*t*, C-8), 29.8 (*t*, C-6), 21.2 (*t*, C-7), 21.2 (*t*, C-3), 18.6 (*t*, C-11), 13.8 (*q*, C-12); EI-MS, *m/z* (rel. int.): 214 $[M]^+$ (1), 213 $[M-H]^+$ (1), 197 $[M-OH]^+$ (100), 185 $[M-H-C_2H_4]^+$ (32), 182 $[M-OH-Me]^+$ (97), 142 $[M-C_2H_4-CO_2]^+$ (85), 127 $[M-C_2H_4-CO_2-Me]^+$ (40), 109 $[M-C_2H_4-CO_2-Me-H_2O]^+$ (66), 99 $[M-C_2H_4-CO_2-C_3H_7]^+$ (43).

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].

Acknowledgements—This research was aided by grants from the Italian Ministry for University and Scientific and Technological Research and in part by the National Research Council of Italy (CNR). The mass spectral data were provided by Servizio di spettrometria di massa del CNR e dell'Università di Napoli Federico II. The assistance of the staff is gratefully acknowledged. Authors thank the Centro Interdipartimentale di Metodologie Chimico-Fisiche dell'Università di Napoli Federico II for NMR. Contribution No. 153 (DISCA).

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