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Chenopodolans A–C: Phytotoxic furopyrans produced by *Phoma chenopodiicola*, a fungal pathogen of *Chenopodium album*

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ABSTRACT

Three tetrasubstituted furopyrans, named chenopodolans A–C, were isolated together with the well known fungal metabolite (−)-(*R*)-6-hydroxymellein from the liquid culture of *Phoma chenopodiicola*, a fungal pathogen proposed for the biological control of *Chenopodium album*, a common worldwide weed of arable crops. The structures of chenopodolans A–C were established by spectroscopic and chemical methods as 2-(3-methoxy-2,6-dimethyl-7a*H*-furo[2,3-*b*]pyran-4-yl)-butane-2,3-diol, 1-(3-methoxy-2,6-dimethyl-7a*H*-furo[2,3-*b*]pyran-4-yl)ethanol and 3-methoxy-2,6-dimethyl-4-(1-methylpropenyl)-7a*H*-furo[2,3-*b*]pyran, respectively. The absolute configuration *R* to the hydroxylated secondary carbon (C-11) of the side chain at C-4 of chenopodolan A was determined by applying an advanced Mosher's method. Assayed by leaf puncture on host and non-host weeds chenopodolans A and B, and the 11-*O*-acetylchenopodolan A showed a strong phytotoxicity. These results showed that the nature of the side chain attached to C-4 is an important feature for the phytotoxicity. A weak zootoxic activity was only showed by chenopodolan B.

1. Introduction

Chenopodium album L., also known as common lambsquarters or fat hen, is a ubiquitous weed of arable crops such as sugar beet and maize (Holm et al., 1977). The difficulties in managing this weed have increased the interest in the use of fungal pathogens as a possible option for its control. *Ascochyta caulina* (P. Karst.) v.d. Aa and v. Kest, has been proposed as a fungal pathogen for the control of *C. album* (Kempenaar, 1995; Kempenaar et al., 1996). More recently another pathogenic member of the Sphaeropsidales, *Phoma chenopodiicola*, has been proposed as a potential biocontrol agent. A new phytotoxic unrearranged *ent*-pimaradiene diterpene, named chenopodolin, was isolated from the liquid culture of this pathogen and characterized as (1*S*,2*S*,3*S*,4*S*,5*S*,9*R*,10*S*,12*S*,13*S*)-1,12-acetoxy-2,3-hydroxy-6-oxopimara-7(8),15-dien-18-oic acid 2,18-lactone (Cimmino et al., 2013). At a concentration of 2 mg/mL, the toxin showed phytotoxic activity, causing necrotic lesions to leaves of *Mercurialis annua*, *Cirsium arvense* and *Setaria viridis*. The bioassay of five prepared derivatives indicated that the hydroxy group at C-3 and the α,β-unsaturated ketone at C-6 are important features for the phytotoxicity, and the acetyl group at C-1 to be not (Cimmino et al., 2013).

Considering that the organic extract obtained from the culture filtrates of the fungus proved to be much more phytotoxic then the expected by the chenopodolin content, it was hypothesized that other compounds could be present in the extract, having additional or synergistic effects.

This manuscript reports on: (a) the isolation and the chemical characterization of three further novel phytotoxic metabolites produced by *P. chenopodiicola*, named chenopodolans A, B and C; (b) the preliminary studies of their biological activities to evaluate their potential as natural and safe herbicides; (c) the absolute configuration of the secondary hydroxylated carbon (C-11) of the side chain at C-4 of chenopodolan A by applying an advanced Mosher's method (Othani et al., 1989).

2. Results and discussion

The organic extract obtained from the liquid culture of *P. chenopodiicola*, having a high phytotoxic activity described below, was purified by CC and TLC detailed in the experimental section. As recently reported, chenopodolin was obtained from fraction 6 of the first chromatographic column as a new unrearranged *ent*-pimaradiene diterpene (Cimmino et al., 2013). Furthermore, a compound was purified from fraction 4, identified as (−)-(*R*)-6-hydroxymellein (**7**, Fig. 1) by its physic (OR) and spectroscopic (^{1}H NMR and ESIMS (+)) data, which were identical to

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those previously reported (Islam et al., 2007). The structure was also supported by data from ESIMS (−) and APCI (+) which showed the pseudomolecular ions at *m*/*z* 193 $[M-H]^-$ and 195 $[M+H]^+$, respectively. The (−)-(*R*)-6-hydroxymellein (**7**) was previously isolated from different fungi, e.g. *Penicillium verruculosum* (Ito et al., 1992) and *Lachnum papyraceum* (Stadler et al., 1995) and also from carrot (Marinelli et al., 1996). The bioassay-guided purification of fractions 1 and 5 of the original column allowed isolation of three further metabolites, two of which have phytotoxic activity, thus confirming the hypothesis that the fungus could produce several bioactive metabolites.

The preliminary ^1H and ^{13}C NMR investigation showed that the three metabolites had similar structures, but very different from that of chenopodolin. Being novel, and belonging to the naturally occurring furopyran group, they were named chenopodolans A, B and C.

Chenopodolan A proved to have the molecular formula $C_{14}H_{20}O_5$, deduced by its HR ESIMS, with five hydrogen deficiencies, and molecular weight of 268. Three of these deficiencies were associated with one tri- and two tetra-substituted conjugated double bonds, as deduced from the preliminary inspection of the ^1H and ^{13}C NMR spectra (Table 1), and in agreement with the typical bands and absorption maxima observed in the IR (Nakanishi and Solomon, 1977) and UV (Scott, 1964) spectra. The other two unsaturations could be attributed to two rings. Indeed, very significant signals were observed in the same spectra at δ 6.21 (s)/92.8 (CH) for a carbon bonded to two oxygen atoms and this represents one of the bridgehead carbons (C-7a) between one pyran and one furan ring. These findings are in agreement with the presence of a olefinic proton (H-5) and its adjacent methyl group (Me-13) belonging to the pyran ring, which coupled (J < 1 Hz) in the COSY spectrum (Berger and Braun, 2004), and resonated as two singlets at the typical chemical shift values of δ 6.59 and 2.21 (Pretsch et al., 2000), respectively. The ^1H NMR spectrum showed also the signals of another methyl (Me-8) and that of a methoxy group resonating both as two singlets at δ 1.94 and 3.91, respectively, and those of a 2,3-dihydroxybutandiol side chain. Indeed, the proton of the secondary hydroxylated carbon (C-11) of this latter appeared as a quartet (J = 6.6 Hz) at δ 3.88 being coupled with the adjacent methyl group (Me-12), which was observed as doublet (J = 6.6 Hz) ad δ 1.20. The methyl group (Me-9) bonded to the quaternary hydroxylated carbon (C-10) of the same residue resonated as a singlet at δ 1.33. The correlations observed in the HSQC spectrum (Berger and Braun, 2004) allowed to assign the chemical shifts of these 7 protonated carbons at δ 137.1, 13.4, 8.8, 56.3, 73.7, 17.4 and 23.2 (C-5, C-13, C-8, OMe, C-11, C-12 and C-9, respectively) (Breitmaier and Voelter, 1987). The other five olefinic carbons, three of which were oxygenated, were assigned on the basis of the correlations observed in the HMBC spectrum (Table 2) (Berger and Braun, 2004). Thus, the signals observed at δ 165.9, 165.0, 160.0, 129.5 and 103.1 were assigned to C-3, C-2, C-6, C-4 and C-3a, respectively. Similarly, the signal at δ 76.1 was assigned to C-10. On the basis of the couplings observed in the HMBC spectrum, C-3a represents the other bridgehead carbon between the pyran and the furan rings (Breitmaier and Voelter, 1987). The couplings observed in the same spectrum between Me-9 and C-5, and between H-5 and C-10 allowed to locate the 2,3-butandiol side chain on C-4. Considering that all the chemical shifts were assigned to protons and carbons of **1,** as reported in Table 1, chenopodolan A could be formulated as 2-(3-methoxy-2,6-dimethyl-7a*H*-furo[2,3-*b*]pyran-4-yl)-butane-2,3-diol (**1**).

The structure assigned to **1** was supported by the data from its HRESI MS and NOESY spectra. The HRESI MS showed the dimeric sodiated form, the potassium and sodium clusters at *m*/*z* 559 $[2M+Na]^+$, 307 $[M+K]^+$, 291.1217 $[M+Na]^+$, respectively. By H_2O loss the sodium cluster produced the ion at *m*/*z* 273. The APCI spectrum showed the pseudomolecular ion at *m*/*z* 269 $[M+H]^+$ which, by loss of H_2O, produced the ion at *m*/*z* 251.

The NOESY spectrum (Table 3) (Berger and Braun, 2004) showed the correlations between the protons of the side chain (H-11, Me-9 and Me-12) with H-5, the correlation between H-7a with both Me-13 and the methoxy group, and that of H-11 with Me-9 and Me-12. Due to these correlations, in agreement with the inspection of a Dreiding model of **1** with the exception of the butandiol side chain at C-4, chenopodolan A appears to have a quite flatted rigid structure.

The structure assigned to **1** was confirmed by preparing the 11-*O*-acetyl derivative (**4**) by routine acetylation with pyridine and acetic anhydride. The IR spectrum of **4** showed the typical band of the acetoxy group together with that of the tertiary hydroxy group. Its ^1H NMR spectrum differed from that of **1** only for the downfield shift of H-11 ($\Delta\delta$ 1.12), always appearing as a quartet (J = 6.4 Hz) at δ 5.00, and for the presence of the singlet of the acetyl group at δ 2.08. Its ESIMS spectrum showed the dimeric sodiated form $[2M+Na]^+$, the potassium $[M+K]^+$ and sodium $[M+Na]^+$ clusters at *m*/*z* 643, 349 and 333, respectively. By loss of H_2O the latter ion generated the peak at *m*/*z* 315. The APCI mass spectrum showed the pseudomolecular ion $[M+H]^+$ at *m*/*z* 311; this latter produced the ions at *m*/*z* 293 and 233, by successive losses of H_2O and AcOH, respectively.

Several attempts to crystallize chenopodolans A, as well as B and C, carried out by changing solvent mixtures and temperatures, failed probably also due to the limited amounts available of the metabolites. Thus, X-ray analysis could not be performed to assign the relative configuration to **1**, **2** and **3**.

For chenopodolan A, the absolute configuration of the secondary hydroxylated carbon (C-11) group of the 2,3-butanediol attached to C-4 was determined by applying an advanced Mosher's method (Othani et al., 1989). By reaction with *R*-(−)-α-methoxy-α-trifluoromethylphenylacetyl (MTPA) and *S*-(+)MTPA chlorides, chenopodolan A (**1**) was converted into the corresponding diaste-

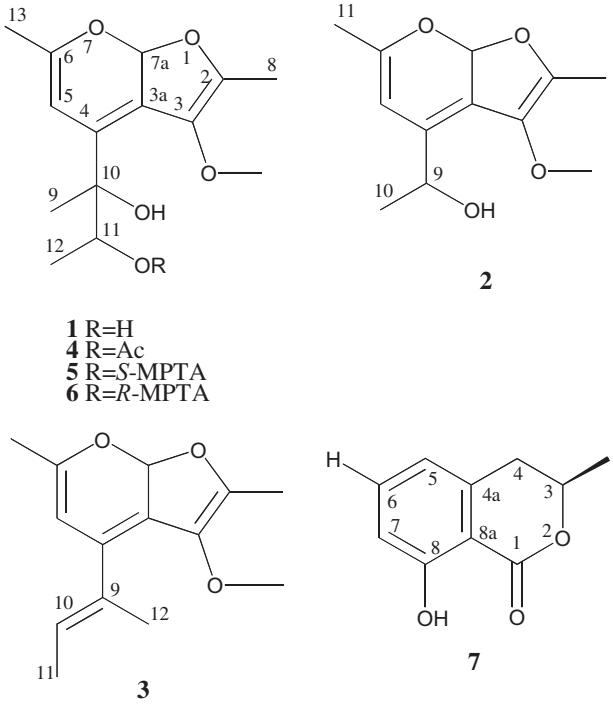

Fig. 1. Structures of: chenopodolans A–C (**1**–**3**, respectively), 11-*O*-acetyl-, and 11-*O*-*S*-MTPA and 11-*O*-*R*-MTPA esters (**4**–**6**, respectively) of chenopodolan A; and (−)-(*R*)-6-hydroxymellein (**7**).

Table 1
^{1}H and ^{13}C NMR data of chenopodolans A–C (**1**–**3**, respectively).[a,b]

Position	**1**		**2**		**3**	
	δC[c]	δH (*J* in Hz)	δC[c]	δH (*J* in Hz)	δC[c]	δH (*J* in Hz)
2	165.0 *s*		164.7 *s*		164.5 *s*	
3	165.9 *s*		165.5 *s*		165.4 *s*	
3a	103.1 *s*		103.1 *s*		101.5 *s*	
4	129.5 *s*		126.8 *s*		123.3 *s*	
5	137.1 *d*	6.59 *s*	136.9 *d*	6.54 *s*	136.2 *d*	7.00 *s*
6	160.0 *s*		159.3 *s*		160.3 *s*	
7a	92.8 *d*	6.21 *s*	92.9 *d*	6.19 *s*	91.8 *d*	6.15 *s*
8	8.8 *q*	1.94 (3H) *s*	8.7 *q*	1.96 (3H) *s*	8.1 *q*	1.83 (3H) *s*
9	23.2 *q*	1.33 (3H) *s*	64.9 *d*	4.72 (1H) *q* (*J* = 6.0 Hz)	132.5 s	
10	76.1 *s*		23.3 *q*	1.35 (3H) d (*J* = 6.0 Hz)	128.6 *d*	5.61 (1H) *q* (*J* = 6.9 Hz)
11	73.7 *s*	3.88 (1H) *q* (*J* = 6.6 Hz)	12.7 *q*	1.95 (3H) *s*	16.5 *q*	1.76 (3H) *d* (*J* = 6.9 Hz)
12	17.4 *q*	1.20 (3H) *d* (*J* = 6.6 Hz)			13.5 *q*	1.94 (3H) *s*
13	13.4 *q*	2.21 (3H) *s*			8.1 *q*	2.03 (3H) s
OMe	56.3 *q*	3.91 *s*	56.1 *q*	3.91 *s*	55.5 *q*	3.91 s

[a] The chemical shifts are in δ values (ppm) from TMS.
[b] 2D ^{1}H, ^{1}H (COSY) ^{13}C, ^{1}H (HSQC) NMR experiments delineated the correlations of all the protons and the corresponding carbons.
[c] Multiplicities were assigned by DEPT spectrum.

Table 2
HMBC data of chenopodolan A–C (**1–3,** respectively).

Carbon	**1**	**2**	**3**
2	Me-8	Me-8	
3	H-7a, OMe	H-7a, OMe	H-7a, OMe
3a	H-7a, Me-8	H-7a, Me-8	H-7a, Me-12
4	H-7a, H-5, Me-13	H-7a, H-9, Me-11	H-5, H-7a, Me-13
5	Me-13, Me-9	Me-11, H-9	H-10, Me-13
6	H-5, H-7a, Me-13	H-5, H-7a, Me-11	H-5, H-7a, Me-13
7a			
8			
9		Me-10	Me-11
10	H-5, Me-9, Me-12		Me-11
11	Me-9, Me-12	H-5	H-10
12			H-5, Me-12

Table 4
^{1}H NMR data of 11'-*O*-(*S*)- and 11'-*O*-(*R*)-MTPA esters of chenopodolan A (**5** and **6**, respectively).

	5 δH (*J* in Hz)	**6** δH (*J* in Hz)
5	6.419 *s*	6.522 *s*
7a	6.162 *s*	6.211 *s*
11	5.238 *q* (*J* = 6.4 Hz)	5.241 *q* (*J* = 6.4 Hz)
OMe	3.905 *s*	3.917 *s*
13	2.135 (3H) *s*	2.198 (3H) *s*
8	1.951 (3H) *s*	1.966 (3H) *s*
9	1.331 (3H) *s*	1.385 (3H) *s*
12	1.381 d (*J* = 6.4 Hz)	1.323 *d* (*J* = 6.4 Hz)
OMe	3.574 *s*	3.525 *s*
Ph	7.72-7.35 *m*	7.72-7.38 *m*

reomeric *S*-MTPA and *R*-MTPA monoesters at C-11 (**5** and **6**, respectively), whose spectroscopic data were consistent with the structure assigned to **1**. Subtracting the chemical shift of the protons (Table 4) of the 11-*O*-*R*-MTPA (**6**) from that of 11-*O*-*S*-MTPA (**5**) esters, the Δδ (**5**–**6**) values for all of the protons were determined as reported in Fig. 2. The positive Δδ values were located on the right-hand side, and those with negative values on the left-hand side of the model A as reported in Othani et al. (1989). This model allowed the assignment of the *R* configuration at C-11. Then **1** was formulated (11*R*) 2-(3-methoxy-2,6-dimethyl-7a*H*-furo[2,3-*b*]pyran-4-yl)-butane-2,3-diol (**1**).

Chenopodolans B and C differed from chenopodolan A for the side chain attached to C-4. In chenopodolan B (molecular weight 224 due to its molecular formula of $C_{12}H_{16}O_4$), this chain is a 1-ethanol, as results by comparison of its ^{1}H and ^{13}C NMR data with those of **1** (Table 1). The data of COSY, HSQC and HMBC (Table 2) spectra of **2**, which showed the signals for the tetrasubstituted furopyran moiety very similar to those of **1**, allowed to locate the side chain at C-4. However, instead of the signals of the 2, 3-butanediol, those typical for the 1-ethanol residue were ob-

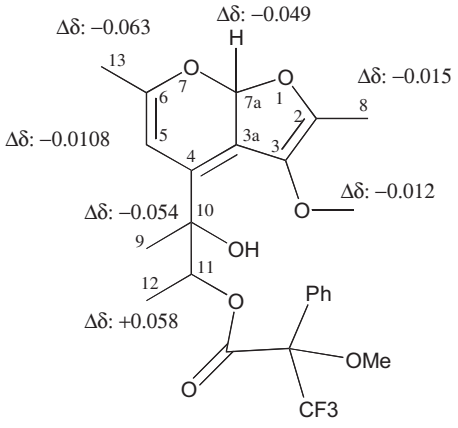

Fig. 2. Structures of 3-*O*-S- and 3-*O*-R-MTPA esters of chenopodolan A (**5** and **6**, respectively), reporting the Δδ value obtained by comparison (**5**-**6**) of each proton system.

Table 3
NOESY data of of chenopodolans **A–C** (**1**–**3**).

1		**2**		**3**	
Irradiated	Observed	Irradiated	Observed	Irradiated	Observed
H-5	Me-9, H-11, Me-12	H-7a	OMe, Me-11	H-5	Me-11
H-7a	M-13, OMe	H-5	Me-10	H-7a	OMe, Me-13
H-11	Me-9, Me-12	H-9	Me-10, Me-11	H-10	H-5, Me-11

served at δ 4.72 (1H, q, J = 6.0 Hz)/64.9 (CH-9) and 1.35 (3H, d, J = 6.0 Hz)/23.3 (Me-10). Considering all the chemical shifts assigned to the protons and the carbons of **2** (Table 1), chenopodolan B could be formulated as 1-(3-methoxy-2,6-dimethyl-7a*H*-furo[2,3-*b*]pyran-4-yl)ethanol.

The structure assigned to **2** was supported by the data from its HRESI MS and NOESY spectra. The HRESI MS spectrum showed the dimeric sodiated form $[2M+Na]^+$, the potassium$[M+K]^+$ and sodium $[M+Na]^+$ clusters at m/z 471, 263, 247.0934, respectively. By loss of H_2O the latter generated the ion at m/z 229. The APCI mass spectrum showed the pseudomolecular ion $[M+H]^+$ at m/z 225 which, by loss of H_2O, generated the ion at m/z 207.

The NOESY spectrum (Table 3) showed correlations of the proton H-5 with Me-10, H-7a with Me-11 and OMe, and H-9 with Me-10 and Me-11. These results, similarly to **1** and in agreement with the inspection of a Dreiding model, showed a enough flatted rigid structure with the exception of the ethanol side chain at C-4.

It was not possible to assign the absolute configuration to the secondary hydroxylated carbon (C-9) of the ethanol residue of **2** because all the attempts made to convert it into the corresponding diastereomeric *S*-MTPA and *R*-MTPA esters, by reaction with *R*-(−)-α-methoxy-α-trifluoromethylphenylacetyl (MTPA) and *S*-(+)MTPA chlorides, as above reported for chenopodolan A (**1**), failed. This probably could be a consequence of a steric hindrance due to the bulky of the reagent group and to the proximity of the ethanol residue to the furopyran ring system.

In chenopodolan C (**3**), which showed a molecular weight of 234 due to its molecular formula of $C_{14}H_{18}O_3$, the side chain was a 1-methylpropenyl residue. The comparison of its ^1H and ^{13}C NMR data (Table 1), based also on the COSY, HSQC and HMBC (Table 2) couplings with those of **1** showed that the signals of the same tetrasubstituted furopyran moiety were very similar and supported the location of the side chain at C-4. Furthermore, the signals of the 2,3-butanediol were absent in the ^1H NMR spectrum, while those for a 1-methylpropenyl residue side chain were observed. In particular, the olefinic proton (H-10) and its adjacent methyl group (Me-11) resonated as quartet (J = 6.9 Hz) and a doublet (J = 6.9 Hz) at δ 5.61 and 1.76, while the other methyl group resonated as singlet at δ 1.94 (Me-12). The corresponding carbons in the ^{13}C NMR spectrum were observed at δ 128.6, 16.5 and 13.5 (C-10, C-11 and C-12, respectively) while the quaternary olefinic carbon C-9 resonated at δ 132.5.

Considering all the chemical shifts assigned to protons and carbons of **3** (Table 1) chenopodolan C could be formulated as 3-methoxy-2,6-dimethyl-4-(1-methylpropenyl)-7a*H*-furo[2,3-*b*]pyran.

The structure assigned to **3** was supported by the data from its HRESI MS and NOESY spectra. The HRESI MS spectrum showed the trimeric $[3M+Na]^+$ and dimeric $[2M+Na]^+$ sodiated form, the potassium $[M+K]^+$ and sodium $[M+Na]^+$clusters, and the pseudomolecular ion $[M+H]^+$ at m/z 725, 491, 273, 257.1164 and 235.1343, respectively.

The NOESY spectrum (Table 3) showed the correlations between the proton H-5 with those of Me-11, H-7a with both Me-13 and the methoxy group, and H-10 with H-5 and Me-11. The lacking of a NOE correlation between H-10 and Me-12 allowed to assign a *E*-stereochemistry to the double bond of the side chain at C-4. Furthermore, as already reported for **1** and **2**, and in agreement with the inspection of a Dreiding model, these results confirm for **3** a quite flatted rigid structure, with the exception of the 1-methylpropenyl side chain at C-4.

Assayed on punctured detached leaves on *Sonchus oleraceus*, *M. annua* and *C. album*, chenopodolan B proved to be the most effective, causing spread necrosis on all the three plant species tested (level 4; data not shown). Chenopodolan A and 11-*O*-acetylchenopodolan A proved to have almost the same toxicity, being active in particular against *S. oleraceus* and *M. annua*. Chenopodolan C was completely inactive (data not shown). These results showed that the nature of the side chain attached to C-4 is an important feature for the phytotoxicity. In particular, the presence of the tertiary hydroxy group in **1** (C-10), which become secondary in **2** (C-9) is very important for the activity. **3** was completely inactive and this last result probably is due to the different side chain at C-4 being a 1-methyl propenyl. The activity of the 11-*O*-acetylchenopodolan A (**4**) further supported this result, because in this derivative the secondary hydroxy group was acetylated only at C-11 whilst it is free at C-10. Compound **7** proved to have almost the same toxicity of **1** and **2**.

In the assay for zootoxicity (*Artemia salina*), only chenopodolan B was weakly active, causing around 24% larval mortality after 48 h of exposure to the metabolite (data not shown). All the other compounds were inactive. Assayed on fungi (*Geotrichum candidum*) and gram positive (*Bacillus subtilis*) and gram negative (*Escherichia coli*), none of the metabolites showed antimicrobial activities (data not shown).

In conclusion, three novel furopyrans, named chenopodolans A–C, were isolated from *P. chenopodiicola*, a fungus proposed for the biocontrol of *C. album*. Chenopodolans A and B could have additive phytotoxic activities with chenopodolin and (−)-(*R*)-6-hydroxymellein produced by the same fungus. Furopyrans are very rare as naturally occurring compounds. The only example of this type is aracemosolone, isolated from root bark of *Bauhinia racemosa* Lamk (Renuka et al., in press). Some heteroannelated furopyrans were synthesized as herbicides (Kiyoshi et al., 1997). Thus, considering the unusual structures associated to the preliminary biological activities, it seems the new compounds would deserve further attentions for better understanding their biological properties and to evaluate their potential as lead for novel herbicides of natural origin.

3. Experimental

3.1. General

Optical rotation was measured in $CHCl_3$ on a Jasco P-1010 digital polarimeter unless otherwise noted; IR spectra were recorded as glassy film on a Perkin-Elmer Spectrum One FT-IR Spectrometer and UV spectra were recorded in MeOH solution on a Perkin-Elmer Lambda 25 UV/Vis spectrophotometer. ^1H and ^{13}C NMR spectra were recorded at 400/100 MHz in $CDCl_3$ on Bruker spectrometers. The same solvent was used as internal standard. Carbon multiplicities were determined by DEPT spectra (Berger and Braun, 2004). DEPT, COSY-45, HSQC, HMBC and NOESY experiments (Berger and Braun, 2004) were performed using Bruker microprograms. HRESI and ESI and APCIMS spectra were recorded on Waters Micromass Q-TOF Micro and Agilent Technologies 6120 Quadrupole LC/MS instruments, respectively. Analytical and preparative TLC were performed on silica gel plates (Merck, Kieselgel 60 F_{254}, 0.25); the spots were visualized by exposure to UV light and/or by spraying first with 10% H_2SO_4 in MeOH and then with 5% phosphomolybdic acid in EtOH, followed by heating at 110 °C for 10 min. Column chromatography was performed on silica gel (Merck, Kieselgel 60, 0.063–0.200 mm). Solvent systems: (A) $CHCl_3$–*i*-PrOH (95:5); (B) $CHCl_3$; (C) *n*-hexane–Me_2CO (6:4); (D) $CHCl_3$–*i*PrOH (97:3).

3.2. Fungal strain

The fungus was isolated from diseased leaves of *C. album* and identified as *P. chenopodiicola* (Berestetskiy, unpublished). A monoconidial isolate was deposited in the culture collection of both the All-Russian Research Institute of Plant Protection, Pushkin, Saint-Petersburg, Russia (I-13.2) and the Institute of Science of

Food Production, Bari, Italy (ITEM 12534). The isolate was routinely grown and maintained in plates and slants containing potato-dextrose agar (PDA, Sigma–Aldrich, Chemie Gmbh, Buchs, Switzerland).

3.3. Production, extraction, and purification of chenopodolans A–C (**1**–**3**)

The fungus was grown in 1 L Erlenmeyer flasks containing 300 mL of a defined mineral (Pinkerton and Strobel, 1976) as recently described (Cimmino et al., 2013). The material obtained by lyophilising the culture filtrates (4.6 L) of the fungus was extracted by EtOAc (4 × 1 L); the organic extract (1.04 g), having high phytotoxic activity, was fractionated by CC eluted with the solvent system A, as already described (Cimmino et al., 2013). Ten homogeneous fraction groups were collected and groups from 1 to 6 proved to be phytotoxic. The purification of the residue of fraction 6 (62 mg) led to obtain chenopodolin. The residue (39 mg) of fraction 5 of the original column was further purified by preparative TLC, eluted with solvent system A, yielding two homogenous solid compounds that, being two new furopyrans, were named chenopodolan A and B (**1**, *Rf* 0.39, 5.6 mg, 1.2 mg/L, **2**, *Rf* 0.53, 3.1 mg, 0.7 mg/L, respectively). The purification by TLC of the residue (13.7 mg) of fraction 4 (eluent system A) yielded a white amorphous solid (**7**, *Rf* 0.67, 8.7 mg, 1.9 mg/L) which was identified as (−)-(*R*)-6-hydroxymellein, as reported below. The purification of the residue of fraction 1 (30 mg) by preparative TLC, eluted with the solvent system B, yielded a yellow homogeneous solid; being a new furopyran it was named chenopodolan C (**3**, *Rf* 0.45, 10.4 mg, 2.3 mg/L).

3.4. Chenopodolan A (**1**)

Compound **1**: $[\alpha]^{25}_{D}$: +8.5 (*c* = 0.24); IRν_{max} 3407, 1679, 1618, 1555, 1456 cm^{-1}; UV λ_{max} nm ($\log\varepsilon$) 307 (3.7), 214 (4.2); ^{1}H and ^{13}C NMR spectra: see Table 1; HRESI MS (+) *m*/*z*: 559 $[2M+Na]^+$, 307 $[M+K]^+$, 291.1217 $[M+Na]^+$ (calcd for $C_{14}H_{20}NaO_5$ 291.1208), 273 $[M+Na-H_2O]$+; APCIMS (+) *m*/*z*: 269 $[M+H]^+$, 251 $[M+H-H_2O]^+$.

3.5. Chenopodolan B (**2**)

Compound **2:** $[\alpha]^{25}_{D}$: +8.6 (*c* = 0.20); IR ν_{max} 3417, 1673, 1610, 1547, 1454 cm^{-1}; UV λ_{max} nm ($\log\varepsilon$) 303 (3.8), 214 (4.3); ^{1}H and ^{13}C NMR spectra: see Table 1; HRESI MS (+) *m*/*z*: 471 $[2M+Na]^+$, 263 $[M+K]^+$, 247.0934 $[M+Na]^+$ (calcd for $C_{12}H_{16}NaO_4$ 247.0946), 229 $[M+Na-H_2O]^+$; APCI *m*/*z*: 225 $[M+H]^+$, 207 $[M+H-H_2O]^+$.

3.6. Chenopodolan C (**3**)

Compound **3**: $[\alpha]^{25}_{D}$: +4.6 (*c* = 0.20); IR ν_{max} 1673, 1626, 1607, 1547 cm^{-1}; UV λ_{max} nm (log ε) 336 (3.6), 253 (3.7); ^{1}H and ^{13}C NMR spectra: see Table 1; HRESI MS (+) *m*/*z*: 725 $[3M+Na]^+$, 491 $[2M+Na]^+$, 273 $[M+K]^+$, 257.1164 $[M+Na]^+$ (calcd for $C_{14}H_{18}NaO_3$ 257.1154), 235.1343 $[M+H]^+$ (calcd for $C_{14}H_{19}O_3$ 235.1334).

3.7. 11-*O*-Acetylchenopodolan A (**4**)

Chenopodolan A (**1**, 2.5 mg) was acetylated with pyridine (50 μL) and Ac_2O (50 μL) at room temperature for 1 h. The reaction was stopped by addition of MeOH and the azeotrope, obtained by the addition of benzene, was evaporated by an N_2 stream. The oily residue (5.0 mg) was purified by preparative TLC eluted with the solvent system C, to give the 11-*O*-acetyl derivative **4** of chenopodolan A as a homogeneous compound (R_f 0.62, 2.4 mg). Derivative **4** had: IR ν_{max} 3423, 1733, 1676, 1615, 1552, cm^{-1}; UV λ_{max} nm ($\log\varepsilon$): 328 (3.8), 227 (4.3); ^{1}H NMR spectrum, δ 6.51 (s, H-5), 6.21 (s, H-7a), 5.00 (q, *J* = 6.4 Hz, H-11), 3.91 (s, OMe), 2.20 (s, Me-13), 2.08 (s, MeCO), 1.94 (s, Me-8), 1.39 (s, Me-9), 1.26 (d, *J* = 6.4 Hz, Me-12); ESIMS (+) *m*/*z*: 643 $[2M+Na]^+$, 349 $[M+K]^+$, 333 $[M+Na]^+$, 315 $[M+Na-H_2O]^+$; APCIMS (+) *m*/*z*: 311 $[M+H]^+$, 293 $[M+H-H_2O]^+$, 233 $[M+H-AcOH]^+$.

3.8. 11-*O*-(*S*)-α-methoxy-α-trifluoromethyl-α-phenylacetate (MTPA) ester of **1** (**5**)

(*R*)-(−)-MPTA-Cl (20 μL) was added to **1** (1.0 mg) dissolved in dry pyridine (20 μL). The mixture was kept at room temperature for 1 h and then the reaction stopped by adding MeOH. Pyridine was removed by an N_2 stream. The residue (2.5 mg) was purified by preparative TLC, eluted with solvent system D, yielding **5** as a homogeneous oil (R_f 0.31, 1.1 mg). It had: IR ν_{max} 3456, 1725, 1679, 1657, 1550, 1275 cm^{-1}; UV λ_{max} nm ($\log\varepsilon$) 271 (3.34) 224 (sh); ^{1}H NMR spectrum see Table 4; ESIMS (+) *m*/*z* 507 $[M+Na]^+$.

3.9. 11-*O*-(*R*)-α-methoxy-α-trifluoromethyl-α-phenylacetate (MTPA) ester of **1** (**6**)

(*S*)-(+)-MPTA-Cl (20 μL) was added to **1** (1.0 mg) dissolved in dry pyridine (20 μL). The reaction was carried out under the same conditions used for preparing **5** from **1**. The purification of the crude residue (2.2 mg) by preparative TLC eluted with solvent system D, allowed to obtain **6** as a homogeneous oil (R_f 0.31, 1.2 mg). It had: IR ν_{max} 3472, 1728, 1676, 1657, 1597, 1273 cm^{-1}; UV λ_{max} nm ($\log\varepsilon$) 270 (3.31), 225 (sh); ^{1}H NMR spectrum see Table 4; ESIMS (+) *m*/*z* 507 $[M+Na]^+$.

3.10. (−)-(*R*)-6-hydroxymellein (**7**)

Compound **7**, had: $[\alpha]^{25}_{D}$: −40.0 (*c* = 0.4, MeOH); IRν_{max} 3544, 1664, 1625, 1585, 1540, 1251 cm^{-1} [lit, Islam et al., 2007: $[\alpha]^{18}_{D}$: −51 (*c* = 0.10, MeOH); IR (KBr) ν = 3600–2800, 1651, 1632, 1587, 1503, 1477, 1386, 1290, 1257, 1221, 1195, 1170, 1120, 1067, 854, 796, 737 cm^{-1}]; ^{1}H NMR spectrum is very similar to that previously reported and recorded (Islam et al., 2007) in the same conditions, except for the following signals, δ: 2.88 (1H, dd, *J* = 16.0 and 10.0 Hz, H-4A), 2.84 (1H, dd, *J* = 16.0 and 6.0 Hz, H-4B); ESIMS (+) *m*/*z* 217 $[M+Na]^+$; ESIMS (−) *m*/*z* 193 $[M-H]^-$; APCIMS (+) *m*/*z* 195 $[M+H]^+$.

3.11. Biological activities

Each metabolite was first dissolved in a minimum amount of MeOH (10^{-1} M or not higher than 2% in the final solutions) and then diluted with distilled water to the desired concentrations. The following bioassays were performed:

Leaf puncture assay: The compounds were tested by using a leaf puncture assay on 3 plant species (*C. album* L., *M. annua* L., and *S. oleraceus* L.) (Evidente et al., 2005). Pure compounds were tested at 6.85×10^{-3} M by applying a droplet (20 μl, 2% MeOH) of solution to detached leaves previously punctured with a needle. Five replications (droplets) on separate leaves were used for each metabolite and for each plant species tested. Leaves were kept in a moistened chamber under continuous fluorescent lights. Symptoms were estimated visually between 3 and 5 days after droplet application, by using a visual scale from 0 (no symptoms) to 4 (necrosis wider than 1 cm). Control treatments were carried out by applying droplets not containing the metabolites.

Antimicrobial bioassay: The antimicrobial activity was tested against three microorganisms by using an agar diffusion assay according to the protocol previously described (Bottalico et al., 1990). In particular, the antifungal activity was tested on

G. candidum (ITEM 781) grown on PDA, whereas the antibiotic activity was assayed against *B. subtilis* (strain S106.2) (a gram positive bacterium) grown on TGYA (tryptic glucose yeast agar – Biolife, Bothell, WA) and *E. coli* (ATCC 29425) (a gram negative bacterium), grown on LB agar (Sigma, St. Louis, MO). Up to 50 μg of each metabolite was applied per diskette. Three replications were performed for each compound. The eventual presence of an inhibition halo of the microbial growth was visually assessed 1 day after the application.

Zootoxic activity assay: The zootoxic activity was evaluated on *A. salina* L. larvae (brine shrimps) by using the protocol already described (Bottalico et al., 1990). The metabolites were tested at 1.7×10^{-4} M, with four replications for each compound. Forty-eight hours after assay performing, the number of dead larvae was counted, and toxicity was then expressed as percentage of dead larvae referred to the total.

Statistical analysis: All the bioassays were performed twice with at least 3 replicates. When appropriate, standard deviation was determined.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.phytochem.2013.10.007.

References

Berger, S., Braun, S., 2004. 200 and More Basic NMR Experiments: A Practical Course, first ed. Weinheim, Wiley-VCH.

Bottalico, A., Capasso, R., Evidente, A., Randazzo, G., Vurro, M., 1990. Cytochalasins: structure–activity relationship. Phytochemistry 29, 93–96.

Breitmaier, E., Voelter, W., 1987. Carbon-13 NMR Spectroscopy. VCH, Weinheim, pp. 183–280.

Cimmino, A., Andolfi, A., Zonno, M.C., Avolio, F., Santini, A., Tuzi, A., Berestetskyi, A., Vurro, M., Evidente, A., 2013. Chenopodolin: a phytotoxic unrearranged ent-pimaradiene diterpene produced by *Phoma chenopodicola*, a fungal pathogen for *Chenopodium album* biocontrol. J. Nat. Prod. 76, 1291–1297.

Evidente, A., Andolfi, A., Vurro, M., Fracchiolla, M., Zonno, M.C., Motta, A., 2005. Drazepinone a tetrasubstituted tetrahydronaphthofuroazepinone with herbicidal activity produced by *Dreschlera siccans*. Phytochemistry 66, 715–721.

Holm, L.G., Pluckett, D.L., Pancho, J.V., Herberger, J.P., 1977. The World's Worst Weeds (Distribution and Biology). University Press of Hawaii, Honoloulou, pp. 84–91.

Islam, S.Md., Ishigami, K., Watanabe, H., 2007. Synthesis of (−)-mellein, (+)-ramulosin, and related natural products. Tetrahedron 63, 1074–1079.

Ito, M., Maruhashi, M., Sakai, N., Mizoue, K., Hanada, K., 1992. NG-011 and NG-012, novel potentiators of nerv growth factor. I. Taxononomy, isolation, and physicochemical and biological properties. J. Antibiot. 45, 1559–1615.

Kempenaar, C., 1995. Studies on the Biological Control of *Chenopodium album* by *Ascochyta caulina*, Doctoral Thesis. University of Wageningen, The Netherlands.

Kempenaar, C., Horsten, P.J.F.M., Scheepens, P.C., 1996. Spore germination and disease development of *Chenopodium album* after application of *Ascochyta caulina* as a mycoherbicide. Eur. J. Plant Pathol. 102, 143–153.

Kiyoshi, A., Fumiaki, K., Hiroyuki, S., Takeshi, K., Sadafumi, K., 1997. Eur. Pat. Appl., EP 755935 A1 19970129.

Marinelli, F., Zanelli, U., Ronchi, V.N., 1996. Toxicity of 6-methoxymellein and 6-hydroxymellein to the producing carrot cells. Phytochemistry 42, 641–643.

Nakanishi, K., Solomon, P.H., 1977. Infrared Absorption Spectroscopy, second ed. Holden Day, Oakland, pp. 17–44.

Othani, I., Kusumi, T., Ishitsuka, M.O., Kakisawa, H., 1989. An application of an advanced Mosher's method. Tetrahedron 30, 3147–3150.

Pinkerton, F., Strobel, G.A., 1976. Serinol as an activator of toxin production in attenuated cultures of *Heliminthosporium sacchari*. Proc. Natl. Acad. Sci. U.S.A. 73, 4007–4011.

Pretsch, E., Bühlmann, P., Affolter, C., 2000. Structure Determination of Organic Compounds – Tables of Spectral Data. Springer-Verlag, Berlin, pp. 161–243.

Renuka, J., Namita, Y., Teena, B., Satish, J., in press. A new pentacyclic phenol and other constituents from the rot bark of *Bauhinia racemosa* Lamk Nat. Prod. Res. http://dx.doi.org/10.1080/14786419.2013.771351.

Scott, A.I., 1964. Interpretation of Ultraviolet Spectra of Natural Products. Pergamon Press LTD, Oxford, pp. 15–162.

Stadler, M., Anke, H., Sterner, O., 1995. Metabolites with nematicidal and antimicrobial activities from the ascomycete *Lachnum papyraceum* (Karst) Karst. III. Production of novel isocoumarin derivatives isolation and biological activities. J. Antibiot. 48, 261–266.