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A New Fungal Growth Inhibitor from *Trichoderma viride*

Luisa Mannina*, Anna Laura Segre

Istituto di Strutturistica Chimica and NMR Service, C.P. 10,

00016 Monterotondo Scalo, Roma, Italy.

Alberto Ritieni, Vincenzo Fogliano, Francesco Vinale, Giacomino Randazzo

Dipartimento di Scienza degli Alimenti, Università di Napoli "Federico II".

Lucia Maddau, Antonio Bottalico

Istituto di Patologia Vegetale, Università di Sassari.

Abstract: A new tetracyclic diterpene having molecular formula $C_{20}H_{28}O_2$ and molecular mass of 300 a.m.u., has been isolated from culture filtrate of a strain of Trichoderma viride. The pure compound shows antifungal activity; its structure elucidation was accomplished using 2D-NMR strategies and chemical derivatization.

INTRODUCTION

Trichoderma spp. are well known for their antagonistic ability towards plant pathogenic fungi and they have received considerable attention as biocontrol agents of siol-borne plant pathogens.[1]

Certain *Trichoderma* strains are known to produce different classes of bioactive metabolites including antibiotics such as peptaiboils, and inhibitors of fungal growth mainly of a terpenic nature.[2]

Circumstantial evidence implicating the action of these metabolites as a contributing mechanism in biocintrol is increasing.

Preliminary investigation on an isolate of *T. viride*, which shows strong antifungal activity without phytopathogenic effects led to the identification of a metabolite able to inhibit fungal growth.

The aim of this work is to elucidate the structure of this active metabolite, in order to clarify its role as a growth inhibitor of pathogenic fungi, and to evaluate the potential use of *T. viride* as a possible agent for biocontrol in agriculture.

RESULTS AND DISCUSSION

Culture filtrates of *T. viride* are known for their strong antifungal activity which does not affect plant development[1,3]. Their antagonistic activity is the result of the sinergic effect of several compounds; in fact, antifungal activity is observed in different fractions of the chromatographic separation. One of the fungal metabolites, which strongly inhibits fungal growth in the *in vitro* biological assay,[3] was extracted in ethyl acetate and purified by conventional chromatography as described in the experimental section. To assess its purity it was analysed by GC-MS and HPLC, with diode array detection. Both liquid and gas chromatograms display a single peak with a retention time of 14.4 min and of 14.9 min, respectively. The MS spectrum of the GC peak (Fig 1) gives a molecular ion with 300 a.m.u., with main fragmentation peaks at 285 a.m.u. (M^+-CH_3) and 272 a.m.u. (M^+-CO). The pure compound has an $[\alpha]^D_{25}$ of +1.17 and its UV spectrum shows a λ_{max} at 255.5 nm with an ε of 1760; the IR spectrum shows strong absorptions at 1731, 1697 and 1661 cm^{-1}, thus suggesting the presence of two carbonyl groups and a double bond, respectively[4]. The presence of two CO groups was confirmed by the $^{13}C\{^1H\}$ spectrum which shows two carbonyl signals at 198.02 and 214.49 ppm. Moreover, two fully substituted olefinic carbons at 146.57 and 149.62 ppm are also present. The other 16 signals of the ^{13}C spectrum are all shifted upfield, with resonances higher than 60 ppm, and thus they belong to saturated carbons. Comparison of the observed $^{13}C\{^1H\}$ spectrum with the ^1H-^{13}C heterocorrelated map revealed the presence of 5 CH_3, 5 CH_2, 3CH, and 3 fully substituted C atoms. The ^1H spectrum shows resonances only in the range 0.98-2.92 ppm. Resonances due to five CH_3 groups can be observed, while peak integration confirms the presence of additional 13 H atoms. From all these results the presence of 18 C, 28 H, and 2 CO can be predicted, suggesting the molecular formula $C_{20}H_{28}O_2$, which results in a molecular mass of 300 a.m.u., in agreement with MS data.

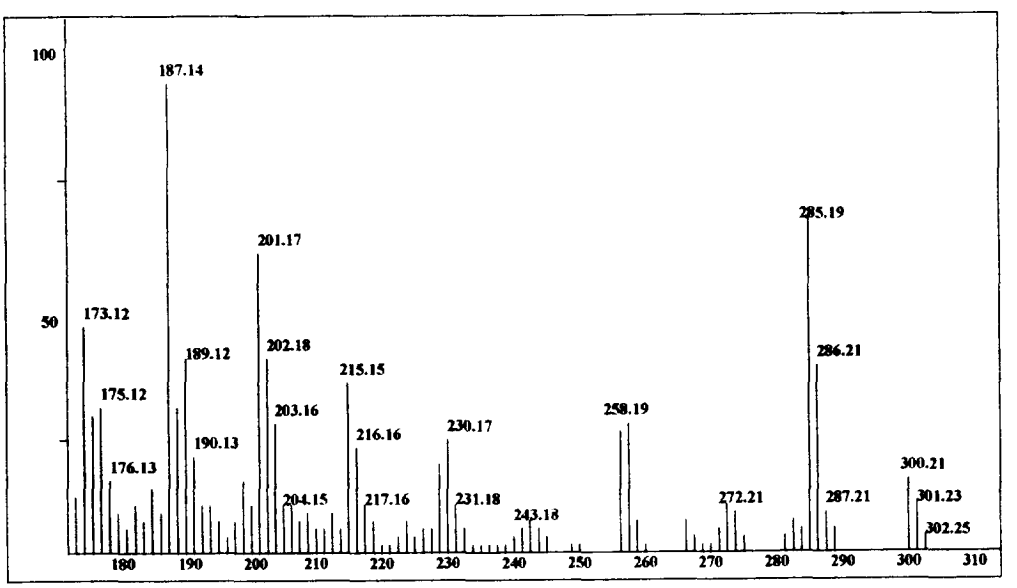

Fig.1. Electronic impact MS spectrum of the GC peak

This molecular formula implies the presence of 7 unsaturations: one of these is the fully substituted double bond; two are due to C=O; therefore four rings must be present. Possible structure models of the unknown compound were obtained by 2D-NMR experiments. The ^{1}H-^{1}H COSY spectrum[5] (Fig.2) allows the identification of the following four independent spin systems indicated by the shaded areas (see Fig 5):

1) ABC X3: 2' (2.08); 2" (2.89); 3' (2.92); 16' (1.11).
2) ABC X: 14' (2.27); 13' (1.52); 13" (2.03); 12' (2.47).
3) ABCD: 5' (1.41); 5" (1.91); 6' (2.03); 6" (2.43).
4) AB: 9' (2.44); 9"(2.57).

All carbons bearing protons atoms were assigned and are reported in Table I using an ^{1}H-^{13}C heterocorrelated experiment (Fig.3) which was obtained with the HMQC sequence (inv4tp) i.e. a 2D heteronuclear shift correlation reverse detected experiment.[6] Since no large spin systems are present in the molecule, the ^{1}H-^{1}H TOCSY[7] (not shown) does not add any other information. Due to the high number of quaternary carbons in the molecule, the structure determination requires further investigation to determine the position of the C=O groups, and the four CH_3 groups which are attached to quaternary carbons. In order to establish the connectivity of the different spin systems and to assign the resonances due to quaternary carbon atoms, a 2D ^{1}H-^{13}C correlation experiment,[8] reverse detected via heteronuclear zero and double quantum coherence, was performed (Fig.4). The used sequence is able to connect quaternary C atoms to any second or third neighbour.

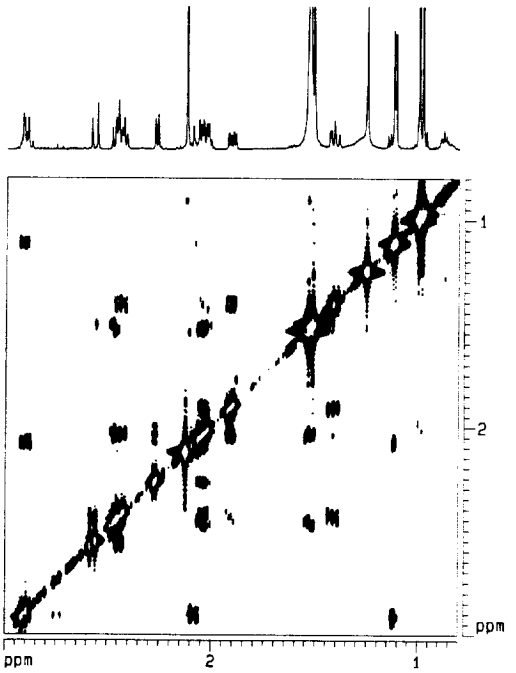

Fig.2. 600.13 ^{1}H-^{1}H COSY map. In F2 dimension ^{1}H spectrum is reported.

Table I. ^{1}H and ^{13}C NMR data of isoharziandione and H_2-isoharziandione

ISOHARZIANDIONE						H_2- ISOHARZIANDIONE				
Cnr.	type	^{13}C (ppm)	^{1}H (ppm)	m.*	*J* (Hz)	type	^{13}C (ppm)	^{1}H (ppm)	m.*	*J* (Hz)
1	CO	214.49				CO				
2	CH_2	42.816	2'= 2.08 2" =2.89	dd m	2"= - 17.0; 3'= 1.9 2'= -17.0; 3'=11.5	CH_2	41.59	2'=2.11 2"=2.86	d dd	2"= -19.3; 2'= -19.3; 3'=11.3
3	CH	30.02	3'= 2.92	m	16'=7.2; 2"=11.5	CH	28.55	3'=2.45	m	2"=11.3
4	C	51.80				C				
5	CH_2	29.89	5'= 1.41 5" =1.91	ddd ddd	5"= -14.1; 6"=12.0; 6'=1.7; 5'= -14.1; 6"= 1.7; 6'=6.3	CH_2	24.05	5'=1.68 5"=1.76	m m	
6	CH_2	29.71	6'= 2.03 6" =2.43	ddd m	6"= -14.0; 5"=6.3; 5'=1.7 6'= -14.0; 5'=12.0; 5"=1.7	CH_2	27.85	6'=1.65 6"=1.76	m m	
7	C	146.57				CH	30.55	7'=2.43	m	17'=7.6
8	C	149.62				CH	72.50	8'=3.09	dd	7'=5.4; 9'=5.4
9	CH_2	60.18	9'=2.44 9"= 2.57	dd dd	9"= - 16.3; 17'=0.7 9'= -16.3; 17'=0.7	CH_2	62.50	9'=2.65 9"=2.71	dd d	9"= -16.7; 8'=5.4 9'= -16.7
10	CO	198.02				CO				
11	C	40.14				C				
12	CH	53.23	12'= 2.47	dd	13'=9.3; 13"=11.3	CH	50.93	12'=2.95	dd	13'=7.6;13"=11.6
13	CH_2	26.85	13'=1.52 13"=2.03	m m	13"=-14.3; 12'=9.3;14'=1.2 13'= -14.3; 12'=11.3;14'=7.9	CH_2	25.38	13'=1.58 13" =2.06	dd m	13"= - 14.2;12'=7.6 13'= - 14.2; 12'=11.6; 14'=7.6
14	CH	59.49	14'=2.27	dd	13"=7.9; 13'=1.2	CH	59.49	14'=2.28	d	13"=7.6
15	C	49.74				C				
16	CH_3	20.89	16'=1.11	d	3'=7.2	CH_3	19.47	16'=1.14	d	3'=7.6
17	CH_3	22.71	17'=2.13	dd	9'=0.7; 9"=0.7	CH_3	15.34	17'=1.14	d	7'=7.6
18	CH_3	20.89	18'=1.52	s		CH_3	24.76	18'=1.39	s	
19	CH_3	25.22	19'=0.98	s		CH_3	21.68	19'=0.99	s	
20	CH_3	23.43	20'=0.99	s		CH_3	23.80	20'=1.05	s	

* multiplicity

The main connections resulting from this 2D experiment are shown by arrows in Fig.5. The analysis of all cross-peaks obtained allows one to link different spin systems through the quaternary carbons; note that cross-peaks due to methyls on quaternary carbons are very informative. From all these considerations two structures may be hypothesized, as reported in Fig 5. In fact, only one proton bearing carbon is present on the four membered ring, so that the exact position of the carbonyl group remains uncertain. This uncertainty is also due to the unknown value of long range coupling costants C-C-C-H and C-C-C-C-H in a planar four membered ring[9]. Thus, the positions of the CH_2 (9) and of C=O (10) can be switched as shown in Fig.5. Moreover, the chemical shift value of CH(3) at 2.89 ppm, is more deshielded than values normally reported. Thus, the possibility that the chemical shift value of this resonance may be influenced by the total three dimensional structure of the molecule was envisaged. A metabolite corresponding to the structure B was elucidated using X-ray diffraction[10] and named Harziandione. However in order to choose between the two structures compatible with our data, a chemical derivatization was considered. For this purpose a most convenient reaction is the hydrogenation of the double bond. In fact, the insertion of a proton into the four membered ring can give the necessary information to assess the molecular structure. Due to the low solubility of our compound in absolute MeOH, the hydrogenation reaction was performed in a mixture of $CHCl_3$ and MeOH. The reaction gave a single product, showing an IR spectrum in which the double bond absorption[4] at 1661 cm^{-1} does not appear any more. This hydrogenated product was purified by TLC and analysed by NMR. All chemical shifts and relative assignments of the hydrogenated derivative are reported in Table I. As expected, in the ^1H spectrum (Fig.6) the signal at 2.13 ppm, previously assigned to CH_3 (17') on the double bond, is upfield shifted to 1.14 ppm.

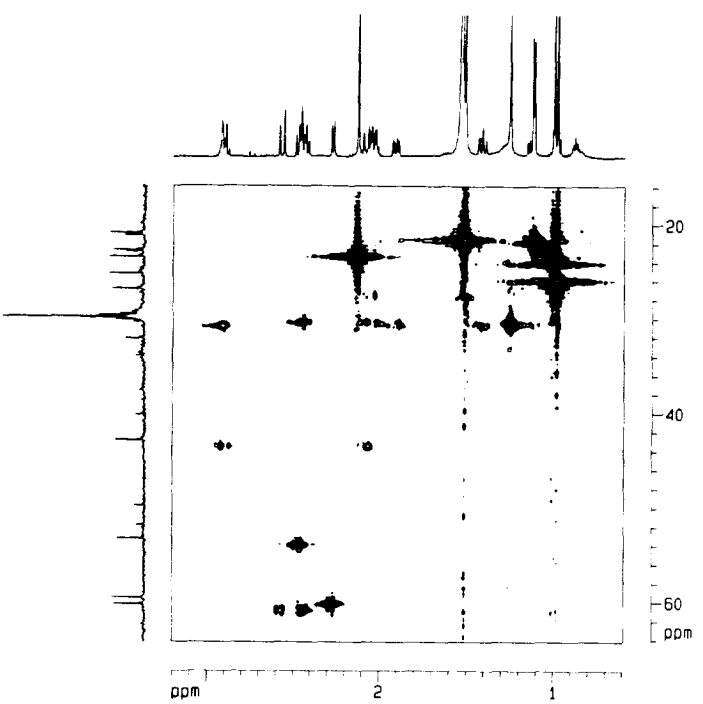

Fig.3. ^1H-^{13}C heterocorrelated map. In F2 dimension ^1H spectrum is reported. In F1 dimension ^{13}C decoupled spectrum is reported

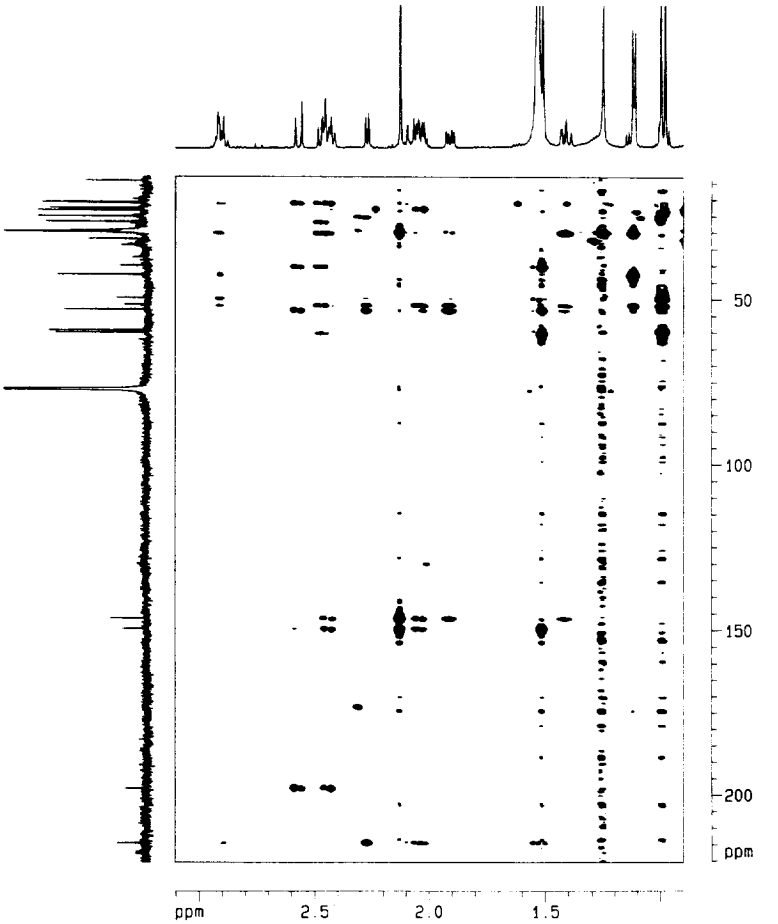

Fig.4. 2D ^{1}H-^{13}C correlation experiment optimized on long range coupling. In F2 and in F1 dimension the ^{1}H spectrum and the ^{13}C decoupled spectrum are reported.

It exactly overlaps the resonance of CH_3 (16'). However, peak integration reveals the presence of six protons. The two methyls at 1.14 ppm are coupled with proton 7' at 2.43 ppm and proton 3' at 2.45, as shown by a ^{1}H-^{1}H COSY 2D map, see Fig.6. The chemical shift of CH (3') has moved from 2.89 ppm to 2.45 ppm. Thus, the hydrogenation induces a large upfield shift in CH(3') confirming that the anomalous chemical shift previously mentioned is due to the large deshielding effect of non-neighbouring atoms.

In the ^{1}H-^{1}H COSY spectrum, a new spin system, due to the two protons inserted by the hydrogenation can be now observed. Protons in positions 7 and 8 have a chemical shift of 2.45 and 3.10 ppm, respectively. The proton in position 8 shows a strong coupling with the two protons at 2.71 and 2.67 ppm, thus indicating that the CH_2 is in position 9 and the carbonyl group is in position 10. The correct structural formula of the new compound is reported in Fig.7, thus confirming the structural formula A of Fig.5. ^{1}H-^{1}H COSY and ^{1}H-^{1}H TOCSY experiments performed on the original compound were also repeated on its hydrogenated derivative; results of these experiments on both molecules are summarized in Table II.

Table II. ^{1}H and ^{13}C correlation of isoharziandione and H_2-isoharziandione

ISOHARZIANDIONE					H_2- ISOHARZIANDIONE		
Cnr.	H	COSY	TOCSY	INV4LPLRND	H	COSY	TOCSY
1				2'; 2"; 3'; 13'; 13"; 14'			
2	2' 2"	2"; 3' 2'; 3'	16' 16'	3'; 14'; 16'	2' 2"	3'; 2" 3'; 2'	16' 16'
3	3'	2'; 2"; 16'		2'; 2"; 5'; 5"; 12'; 16'	3'	2'; 2"; 16'	
4				2'; 2"; 3'; 5'; 5"; 6'; 6";12'; 16'; 19'; 20'			
5	5' 5"	5"; 6"; 6' 5'; 6"; 6'			5' 5"	5"; 6'; 6" 5'; 6'; 6"	17' 17'
6	6' 6"	6"; 5'; 5" 6'; 5'; 5"			6' 6"	6"; 7'; 5'; 5" 6'; 7"; 5'; 5"	17' 17'
7				5'; 5"; 6'; 6"; 17'	7'	8'; 6'; 6"; 17'	
8				6'; 6"; 9"; 9"; 17"; 18'	8'	7' ; 9'	5'; 5"; 6'; 6"; 17'; 18'
9	9' 9"	9" 9'	17'; 18' 17'	12'; 18'	9' 9"	9"; 8' 9'	17'; 18' 17'
10				9'; 9"			
11				9'; 9"; 12'; 13'; 18'			
12	12'	13'; 13"	14'	3'; 5'; 5"; 6'; 6"; 9'; 9";13'; 13"; 14'; 18' ;16'	12'	13'; 13"	14'
13	13' 13"	13"; 12' 13'; 14'	14'	12'	13' 13"	13"; 12' 13'; 14'; 12'	14'
14	14'	13"	12' 13'	13'; 13"; 19"; 20'	14'	13"	12'; 13'
15				3'; 5'; 5"; 13'; 13"; 14'; 19'; 20'			
16	16'	3'	2'	3'	16'	3'	2'; 2"
17	17'		9'; 9"	6'; 6"	17'	7'	5'; 5"; 6'; 6"; 9'; 9"; 8'
18	18'			9'; 9"; 12'	18'	9"	8'; 9'
19	19'			14'; 20'	19'		
20	20'			19'	20'		

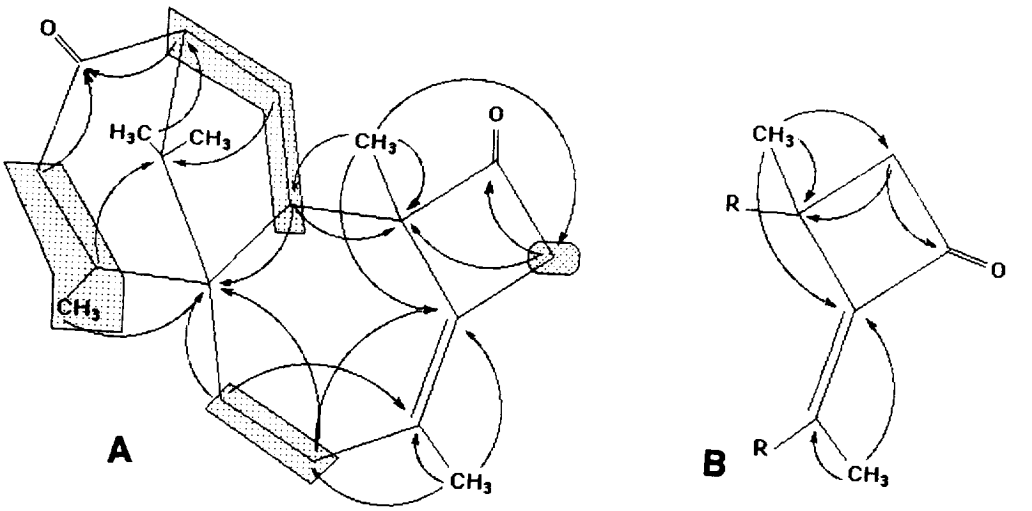

Fig.5. Sketch of the two possible structures.
Arrows indicate the connections resulting from ^{1}H-^{13}C long range coupling experiment.
Dotted areas indicate the four spin systems.

We propose for the newly isolated compound, namely the 3,7,11,15,15 pentamethyl tetracyclo [3-2-1-0^{4-12}-5-2-0^{8-11}]tetradec-7 ene, 1-10 dione, the trivial name of isoharziandione. It is a tetracyclic diterpene. Four isoprenic units are easily identificable; the structure is in full agreement with the biosinthetic isoprenoid rule.

EXPERIMENTAL SECTION

Proton and carbon NMR spectra were run in $CDCl_3$ (1 mg/ml) on a Bruker AMX600 spectrometer operating at 600.13 and 150.92 MHz, respectively. Literature pulse sequences were used for 1D and 2D experiments: ^{1}H-^{1}H COSY: 256x256 data matrix size; time domain (td) 256 in F1 and 512 in F2; relaxation delay (rd)=1.9s; number of scans (ns)=16. Inv4tp: 512X512 data matrix size; td=512 in F1 and 1024 in F2; rd=2s; ns=68; dummy scan (ds) = 4. ^{1}H-^{1}H TOCSY: 512X512 data matrix size; td=512 in F1 and 1024 in F2; rd=2s; ns=32; ds= 4; mixing time= 80ms. Inv4lplrnd: 512X512 data matrix size; td=512 in F1 and 1024 in F2; rd=2s; ds= 16; ns=84; low pass filter 0.00333s; delay for evolution 0.08 s. HPLC chromatography was carried out using a Shimadzu LC10A pump and a Shimadzu Diode array detector using isocratic elution CH_3CN-H_2O (50-50 v/v), with a flow rate of 1 ml/min and Rp18 J&W 5 μm, 250 x 4.6 mm column. GC-MS equipment was a TRIO 2000 Fisons with a Carlo Erba gas chromatograph. The metabolite was eluted with a linear temperature gradient from 80 to 300°C. Low resolution electronic impact spectra were obtained using an ionization energy of 70 eV, 400 mA and a source temperature of 250°C. The IR spectra were recorded on a Perkin-Elmer 399 instrument and UV spectra were measured on a Shimadzu 2100 spectrophotometer, both in $CHCl_3$ solution. The $[\alpha]^{D}_{25}$ was measured by a Jasco DIP-370 digital polarimeter. Analytical and preparative TLC were performed on SiO_2 (Silica gel, Merck plates F_{254}, thickness 0.25 and 0.5 mm).
Identification: *Trichoderma viride* was isolated from soil in Sardinia (Italy). The strain was deposited with number IPVS 1817 at the collection of Istituto di Patologia Vegetale University of Sassary (IPSV).

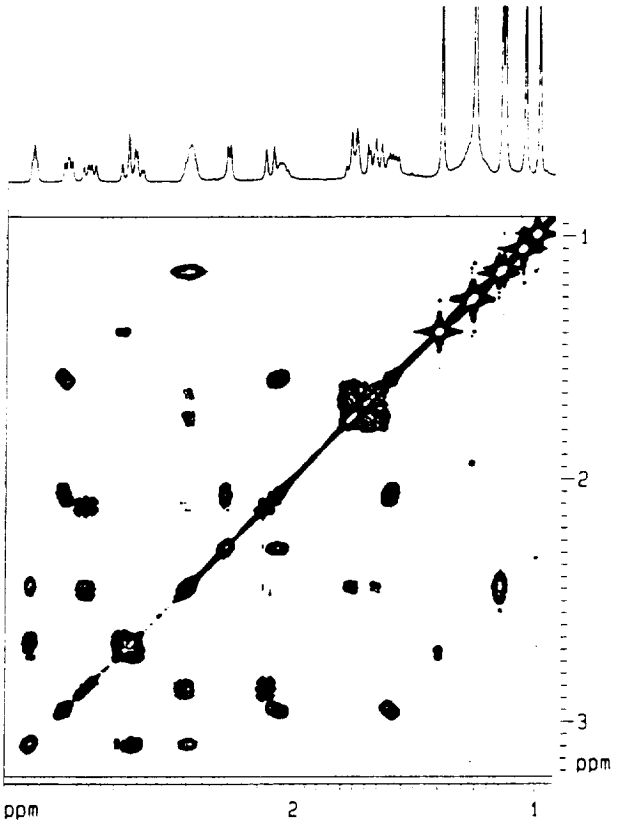

Fig.6. 600.13 ^{1}H-^{1}H COSY map of H_2 derivate. In F2 dimension the ^{1}H spectrum is reported.

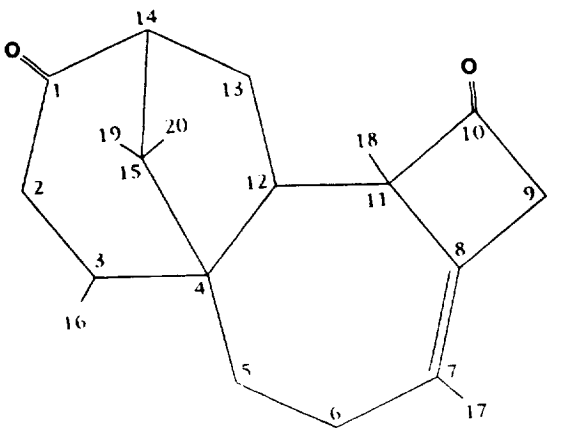

Fig.7. Sketch of structure of isoharzandione. Notation was kept according to the biosynthetic rule.

Isolation procedures: The active metabolite was produced by primary inoculation with *Trichoderma viride* IPSV 1817 in 100 ml of Czapek-Yeast extract. Fermentations were perfomed in Roux flasks for 14 days at 25°C in the dark. The culture filtrates (2,1 l) were concentrated under reduced pressure at 50°C and extracted with ethyl acetate (300 ml for three times) at pH 5. The organic layers were combined then dried on Na_2SO_4 under vacuum at 40°C. The residue (1660 mg) was resuspended in $CHCl_3$-MeOH (80-20 v/v). 50% of crude material (834 mg) and 100% of the antifungal activity was dissolved in this solvent system and applied to a SiO_2 column (600 g, 130 cm high, 40 mm id) which was eluted by the same solvents. The fractions were monitored by TLC eluted by $CHCl_3$-MeOH (95-5 v/v), and visualized by UV light and iodine vapours. The homogeneus fractions were combined, dried and then bioassayed for their bioactivity. Fractions which showed antifungal activity gave a crude residue of 28.9 mg that was further purified by preparative TLC eluted by $CHCl_3$-iPrOH (95-5 v/v). The band at Rf 0.74, detected by fluorescence at 254 nm, was scraped from the plates, extracted with $CHCl_3$, and dried under vacuum. This procedure yielded 9 mg of pure amorphous compound with $[\alpha]^D_{25}$ of +1.17 (c=1 $CHCl_3$); IR ν_{max} at 1731, 1697, 1661, 1602 cm^{-1}; ^1H and ^{13}C NMR see Table 1; eims m/z: 300(25), 285(65), 272(12), 258(19), 230(23), 201(63), 187(100).

Antimicrobial activity: The antimicrobial properties of the fungus metabolites were examined against *Sclerotium rolfsii* by direct application to the inoculum disk surface[3]. The cultures were kept at 25°C and growth rates were recorded for 48 hours.

Syntesis of H_2 derivative: 7.4 mg of pure compound were dissolved in 1 ml of a mixture of $CHCl_3$/MeOH 3:1 and hydrogenated for 24 h using Pd/C 10% as catalyst and an H_2 pressure of 1.2 atm.
The reaction product was purified by TLC (Rf of 0.72 in $CHCl_3$/isopropanol 95/5) giving an amorphous solid that was analysed by NMR. UV spectrum has a λ_{MAX} at 246.5 nm with an ε of 33.4. IR spectrum shows absorption at 1731, 1697, 1602 cm^{-1}; ^1H and ^{13}C NMR see Table 1.

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