

## NOVEL ROLE OF 6-N-PENTYL-6H-PYRAN-2-ONE PRODUCED BY *Trichoderma harzianum* IN HYDROPONIC SYSTEM

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### RESEARCH PAPER

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### ABSTRACT

Secondary metabolites play a pivotal role in the antagonistic activities of some biocontrol species of *Trichoderma* sp. *T. harzianum* used previously as biological control agent against different pathogens. In this work, the effect of *F. oxysporum* f. sp. *gladioli* on the major secondary metabolites secreted by biocontrol strain of *T. harzianum* in Potato Dextrose Broth (PDB) cultures were investigated and quantified; followed by investigating the effect of the previous microorganisms on *T. harzianum* secondary metabolites secreted in *Gladiolus grandiflorus* corms tissues under controlled conditions. Thin Layer Chromatography (TLC), Liquid chromatography Mass spectroscopy (LC/MS), and Nuclear Magnetic Resonance (NMR) techniques were used in this study to determine the major secondary metabolites. This is the first report recording the isolation, characterization and quantification of 6-n-pentyl-6H-pyran-2-one (6PP) and harzianic acid (HA) from the treated corms with *T. harzianum*. The secreted amounts from both metabolites were increased in the pre treated corms with *T. harzianum* followed by infection with *F. oxysporum* f. sp. *gladioli*. Results provided better understandings of the interaction mechanism between *T. harzianum*, *F. oxysporum* f. sp. *gladioli* which could be used in the future in different gene expression studies and will help in using them as bio fertilizers in biocontrol field.

**Key words:** Hydroponic, 6-n-pentyl-6H-pyran-2-one, Harzianic acid, *Trichoderma harzianum*.

### INTRODUCTION

Hydroponic culture became the new modern agricultural technology this years; however the spreading of pathogens considers the major problem especially in closed system technique (Nosir, *et al* 2009). In recent years there has been growing interest in the potential use of microbial metabolites as agrochemicals as an alternative to chemical fungicides. Microbial metabolites may help overcome problems associated with resistance of pathogenic fungi to fungicides and are generally more biodegradable and environmentally friendly than their synthetic counterparts (Tanaka and Mura, 1993). A wide range of antibacterial and antifungal secondary metabolites has been characterised from fungi, including from *Trichoderma* spp. (Vey *et al.*, 2001). The correlation

between the production of antimicrobial metabolites by biological control agents and the effectiveness of Biological control agents (BCA) preparations in vivo is still a matter of conjecture.

*Trichoderma* species are amongst the most common soil-borne fungi and well-known to have biological control capacity against soil-borne plant pathogens (Harman *et al.*, 2004). *Trichoderma* spp. interacts simultaneously with plants and other microorganisms in the rhizosphere, including fungal pathogens. Moreover, they are useful model microorganisms in studies of complex multitrophic plant-microorganism interactions. Modes of action of *Trichoderma* spp. against pathogens include the

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production of antifungal compounds, direct parasitism or inhibition of pathogen growth, cell wall-lytic enzyme activity (Benítez *et al.* 2004; Lorito *et al.*, 1998), competition for nutrients (Chet 1987) and, as determined more recently, induction of systemic and localized resistance in the plant (Harman *et al.*, 2004; Reino *et al.*, 2008).

Secondary metabolites are a heterogeneous group of natural compounds that are considered to aid the producing organism in survival and basic functions, such as competition, symbiosis, metal transport and differentiation (Demain and Fang, 2000).

Species of *Trichoderma* are well-known producers of secondary metabolites with antibiotic activity (Howell, 2004; Demain and Fang, 2000; Ghisalberti and Sivasithamparam, 1998). The natural products produced by *Trichoderma* spp. vary in relation to (1) the specific compound, (2) the isolate and the species of *Trichoderma* involved (3) the presence of other microorganisms in the niche and (4) the balance between elicited biosynthesis and biotransformation rates of the metabolites (Vinale *et al.*, 2009). Secondary metabolites secreted by *Trichoderma* spp. include volatile and non-volatile antifungal substances, such as 6-n-pentyl-6H-pyran-2-one (6PP), gliotoxin, viridin, harzianopyridone, harziandione and peptaibols (Reino *et al.*, 2008). The activities of these metabolites against soilborne plant pathogens have long been studied (Ghisalberti and Sivasithamparam, 1998). Production of secondary metabolites is considered an important factor in biological control, although the mechanisms of action of these compounds in soil and plants are not yet fully elucidated (Demain and Fang, 2000).

The involvement of secondary metabolites produced by *Trichoderma* spp. in the activation of plant defence mechanisms and the regulation of plant

growth was recently investigated, using tomato and oil-seed rape seedlings treated with harzianolide and 6PP isolated from *T. harzianum*, followed by infection with spore suspensions of *Botrytis cinerea* and *Leptosphaeriumaculans*, respectively. In both host plant species, a reduction in disease symptoms was observed, particularly on 6PP-treated plants. Moreover, application of the metabolites led to over-expression of pathogenesis-related (PR) proteins in treated plants (Vinale *et al.*, 2008).

Antibiosis assays demonstrated that secondary metabolites produced by *T. harzianum* (azaphilone, butenolide, harzianolide, harzianopyridone) had different activities towards *Rhizoctoniasolani*, *Pythiummultimum* and *Gaeumannomycesgraminis* var. *tritici* *in vitro* tests, suggesting that individual compounds had specific modes of action (Vinale *et al.*, 2006). The level of production of metabolites by *Trichoderma* spp. varies according to the target pathogen and the strain of *Trichoderma* in use. Increased concentrations of 6PP were secreted by *T. harzianum* in interactions with *Botrytis cinerea*, but the absolute concentration produced was related to the isolate of *Trichoderma* used (Cooney and Lauren, 1998). There are no reports in the literature on the effects of *F. oxysporum* f. sp. *gladioli* on *T. harzianum* natural products.

The aims of the work presented here were to develop an improved understanding of the roles of major secondary metabolites produced by *T. harzianum* in the interaction between *T. harzianum*, *F. oxysporum* f. sp. *gladioli*.

## RESULTS

### Isolation and chemical characterization of major compounds

Accurate mass analysis of compound 1, with [M]<sup>+</sup> at m/z 167.1 (Figure 1) suggested the formula

$C_{10}H_{14}O_2$ , and thus 4 degrees of unsaturation. The  $^{13}C$  NMR spectrum indicated a pyrone carbon at  $\delta 163.0$ , an oxygenated carbon at  $\delta 167.0$ , 3 olefinic carbons ( $\delta 105-140$ ), 4 methylene carbons ( $\delta 20-35$ ) and one methyl carbon at  $\delta 14.0$  (Figure 2 & 3). The UV spectrum of compound 1 had a peak at 336 nm (Figure 4). Database searches indicated that the major unknown compound was 6-n-pentyl-6H-pyran-2-one (6PP). 6PP was the most secondary metabolite in liquid cultures of *T. harzianum*, with a yield of 0.356 mg.l<sup>-1</sup>.

The mass spectrum of compound 2 showed a molecular ion  $[M]^+$  at m/z 366.19 (Figure 5) corresponding to the molecular formula  $C_{19}H_{27}NO_6$ . The structure of compound 2 was deduced from  $^1H$  NMR and  $^{13}C$  NMR spectral data (Figures 6 and 7). The  $^1H$  NMR in  $CD_3OD$  exhibited a doublet at 7.09 ppm (2-H, 7=15Hz), an olefinic proton at 7.53 ppm (3-H) and overlapping signals centered at 6.39 ppm (4-H and 5-H). Analysis of the  $^{13}C$  NMR spectrum of compound 2 suggested that compound 2 had a carboxyl group (178.6 ppm). The UV spectrum of harzianic acid had a peak in absorbance at 259 nm (Figure 8). Database searches and direct comparison with mass and  $^1H$  and  $^{13}C$  NMR spectral data identified the compound as harzianic acid.

#### Production of 6PP and harzianic acid in liquid cultures

*T. harzianum* produced 0.085 mg/ ml 6PP in the liquid medium. In dual cultures with *T. harzianum* and *F. oxysporum* f. sp. *gladioli*, however, 0.125 mg/ml was recovered. Harzianic acid concentrations were nearly doubled in dual cultures of *T. harzianum* and *F. oxysporum* f. sp. *gladioli*, based on peak heights recorded on HPLC.

#### Production of 6PP and harzianic acid in Gladiolus corms

In gladiolus corms treated with *T. Harzianum* alone, 0.058 mg.g<sup>-1</sup> 6PP was found; in contrast, corm tissues treated with *T.harzianum* followed by inoculation with *F. oxysporum* f. sp. *gladioli* had 6-fold greater concentrations of 6PP (0.354 mg.g<sup>-1</sup>) were observed. Harzianic acid concentrations were nearly double in *T. harzianum*-treated and *F. oxysporum* f. sp. *gladioli* inoculated corms, compared with corms treated with *T. Harzianum* alone. Concentrations of HA were estimated based on peak heights recorded on HPLC.

#### Biological activity of 6 PP

*F. oxysporum* f. sp. *gladioli* growth was completely inhibited by 6PP at all concentration from 10  $\mu$ g to 100  $\mu$ g on PDA in Petri dishes.

#### Effect of 6PP on Gladiolus growth in Hydroponic cultures

Gladiolus plant that coated by 10ppm of 6PP germinated 3 days earlier than the untreated and the flower harvest yield was 35 % more than the control in flower fresh weight. Flower stalk diameters in the treated corms with 6PP were 29.5 % taller than the control. The bullet numbers were 46 % more than the untreated. (data not shown).

#### DISCUSSION

The work reported here confirmed the production of antibiotic compounds by *T. harzianum* within corm tissues of Gladiolus infected with *F. oxysporum* f. sp. *gladioli*. Hitherto, the ability of *Trichoderma* spp to secrete antibiotic compounds in infection courts had not been confirmed (Whipps, 2001). Distinguishing the antibiotics secreted in the presence of fungal diseases will help in understanding the mechanisms of action utilized by *T. harzianum* in the biological control of plant pathogens.

The antagonistic mechanisms used by *Trichoderma* are clearly rather complex, and include competitive effects during spore germination and mycelial growth, mediated by the secretion of extracellular enzymes, such as chitinases (Cruz *et al.* 1992),  $\beta$ -glucanases (Lorito *et al.* 1996) and proteinases (Geremia *et al.* 1993), as well as the secondary metabolite produced (Sivasithamparam and Ghisalberti 1998).

The aim of this work was to develop further understanding of the roles of secondary metabolites produced by *T. harzianum* during interactions with *F. oxysporum* f.sp. *gladioli* in interactions with corn tissues of *Gladiolus grandiflorus* tissues. The role of antibiotics produced by *Trichoderma* in biological control remains a matter of conjecture. Although certain metabolites with antibiotic activity may be major factors in the biocontrol activity of a given isolate of the fungus, this may not be the case for other isolates (Harman 2000). The detection of 6PP as the most abundant antifungal compound produced by *T. harzianum* T22 in the presence or absence of *F. oxysporum* f. sp. *gladioli* suggest that this metabolite may be of interest in terms of bio-fungicide potential. Similar results were reported by Vinale *et al.* (2008), who recorded 6PP as a major secondary metabolite secreted by *T. harzianum*, with activity against *B. cinerea* on tomato and *L. maculatum* on oil seed rape. The results obtained in the present work support the suggestion that antibiotic production during saprophytic and antagonistic growth of *T. harzianum* could be involved, in concert with other mechanisms, in the inhibitory interaction with plant pathogens (Howell, 2004).

*F. oxysporum* f. sp. *gladioli* showed greater sensitivity to 6PP than previously reported for *Rhizoctonia solani* and a different *F. oxysporum* (Scarselletti and Faull, 1994). In the earlier work, addition of 300  $\mu\text{g/ml}$  6PP to Potato Dextrose

medium caused a 69.6% growth reduction in *R. solani* and a 31.7% reduction in *F. oxysporum* after 2 days, and completely inhibited the germination of *Fusarium* spores at a concentration of 450  $\mu\text{g/ml}$ . Although, *B. cinerea* metabolized 6PP in agar and on liquid cultures (Cooney and Lauren, 1998). Moreover, a strong relationship was found between the production of 6PP and the antagonistic ability of by *T. harzianum* in vitro and control of *B. cinerea* rots in stored kiwi fruits has also been investigated by Poole *et al.* (1998).

This work findings described in this manuscript confirms Vinale *et al.*, (2008) findings which reveal that the secondary metabolite, 6PP excreted by the antagonistic fungus *T. harzianum* not only interfered with the pathogenic fungus *Botrytis cinerea* or *Leptosphaeria maculans* by inhibiting the mycelial growth and conidia production and germination but by promoting the plant growth and increased the plant resistance against diseases that could be one of the mechanisms *T. harzianum* used to stimulate plant growth. Ghisalberti and Sivasithamparam (1998) previously suggested that some secondary metabolites were directly involved in *T. harzianum*-plant interactions, and that the compound 6PP may act as an auxin-like compound and/or may act as an auxin inducer.

The chemical structures of the *Trichoderma* metabolites isolated in the present work suggests two different possible mechanisms of action. These low molecular weight, non-polar, 6PP and harzianic acid, are produced in high concentrations in the soil environment, and have a relatively long range of influence on the microbial community. In contrast, a short distance effect may result from the presence of polar metabolites and peptides acting in close proximity to the producing hyphae (Lorito *et al.*, 1996).

6PP was recently reported as a plant growth promoting metabolite from *Trichoderma harzianum*



(Vinale *et al.*, 2009), as coating seed of *Brassica napus* with the compound lead to increases in stem length, compared to control seed. In the same work harzianic acid at concentrations as low as 1 – 10 µg L<sup>-1</sup> showed inhibitory activity against *Pythiumirregulare*, *Sclerotiniasclerotiorum* and *Rhizoctoniasolani*.

This work findings and Vinale *et al.*, (2009) findings help in opening a new way in studying the role of metabolites secreted from biocontrol agents in the glasshouse or field studies as the used of use of new products based on biocontrol agents and/or their metabolites for disease control is one of the most promising ways to reduce the dependence on synthetic pesticides in agriculture. Various biocontrol agents have been registered and are available as commercial products, including strains belonging to the genus *Trichoderma* but according to our knowledge there are no registered bio- products from trichoderma metabolites, although Fungal strains of the genus *Trichoderma* are well-known producers of secondary metabolites with antibiotic activity. Their production varies in relation to (i) the specific compound; (ii) the strain and the species; (iii) the presence of other microbes; and (iv) the balance between elicited biosynthesis and biotransformation rtes (Harman, 2004).

Understanding of the roles played by the secondary metabolites of *T. harzianum* may further the development of new, targeted biopesticides and bio-fertilizers based on these naturally occurring compounds. Such compounds may be used as elicitors of plant defense mechanisms, direct toxins to the pathogens and plant growth stimulants.

This is the first work to report the production of secondary metabolites by *T. harzianum* in *Gladiolus* corms infected with *F. oxysporum* f. sp. *Gladioli* in *hydroponics*. The results improve

understanding of the interaction between *T. harzianum* and *F. oxysporum* f. sp. *gladioli* in the host plant tissues. Further studies on the range of antibiotic compounds produced by *T. harzianum* and the efficiency of these compounds in plant-pathogen-antagonist inereactions are required to further increase understanding of the mechanism of action of these bioactive compounds under field conditions.

## Experimental

### Preparation of pathogen inoculum

The inoculum was prepared using an isolate of *F. oxysporum* f. sp. *gladioli* isolated in this work from a purchased *Gladiolus* corm. The culture was maintained on PDA (Oxoid, Basingtoke, Hants, UK) at 22° C and routinely sub-cultured at 15 day intervals. Subcultures of *F. oxysporum* f. sp. *gladioli* were prepared by inoculating PDA with 1 cm diam. disks of colonized PDA plus mycelium, cut from the edge of an actively growing, 7 day old colony.

### Preparation of antagonist inocula

*Trichodermaharzianum* isolate T22, used as a fungal antagonist in this study, was obtained as freeze-dried spores from CentraalbureauvoorSchimmelculturen CBS, The Netherlands. One ml sterilized distilled water was added to the freeze dried spores and 0.1 ml of spore suspension used to inoculate fresh PDA in 9 cm diam. Petri dishes. Cultures were sealed with Parafilm (Alpha Laboratories, Hampshire, UK), and incubated at 22°C with routine sub-culturing at 15 day intervals. Subcultures of *T. harzianum* were prepared by inoculating PDA with 1 cm diam. disks of colonized PDA plus mycelium, cut from the edge of an actively growing, 7 day old colony. Spore suspensions were obtained by flooding 7 day old cultures on PDA with 5 ml sterile distilled water, gently agitating the surface with a wire loop and passing the suspension through two layers of washed sterile muslin directly into 50 ml

centrifuge tubes. Spore suspensions were centrifuged at 3000 rpm (1700 x *g*) in a Thomson–MSE Mistral bench top centrifuge for 10 min. Following rinsing the spore pellets twice in sterile distilled water, with repeated centrifuging (as above); spore concentrations were adjusted to  $4.00 \times 10^8$  spores  $\text{ml}^{-1}$  using repeated hemocytometer counts under a light microscope at a magnification of  $\times 40$ .

#### Inoculation with antagonists

Gladiolus corms, variety Big flower GT01 size 14 (Tylore Bulb, Co., The Netherlands) were surface sterilized in 20 % NaOCl for 20 min before rinsing in running tap water for 6 hours, followed by 3 rinses in sterilized distilled water. Corms were submerged in *T. harzianum* or *A. migulanus* spore suspensions for 30 min. For the interaction treatments, corms were suspended in the antagonist suspension and gently blotted dry on sterilized Whatman, No 3 filter paper under aseptic conditions in a laminar flow cabinet. The combination between *T. harzianum* and *A. migulanus* was prepared by mixing equal volumes of antagonist suspensions in a 2000 ml beaker, immersing surface sterilized corms in the mixed suspension for 30 min and inoculating with the pathogen, as described below. Control corms were immersed in sterilized distilled water for the same length of time.

#### Inoculation with *Fusarium oxysporum* f. sp. *gladioli*

The Gladiolus corms inoculated with antagonists were subsequently inoculated with *F. oxysporum* f. sp. *gladioli* by removing a 10 mm diam., 5 mm deep piece of tissue from the surface of the corm and replacing it with a plug of PDA plus fungal mycelium of the same dimensions. The corms after inoculation were incubated in 22° in laminar flow. Lesion areas developing on inoculated corms were measured 3 days after treatment to estimate the efficiency of the antagonists before collecting the samples for analysis.

#### Antagonist and pathogen culture

*T. harzianum* alone; *T. harzianum* + *F. oxysporum* f. sp. *gladioli* *T. harzianum* treatments (1 L each); was cultured and the secondary metabolites extracted using the method of **Vinale et al., (2006; 2008)**. Two 10 mm diameter plugs of *T. harzianum* T22 were taken from the actively growing margin of cultures on potato dextrose agar (PDA; Oxoid, Basingtoke, Hants, UK) cultures and inoculated into 250 ml conical flasks containing 100 ml of full strength potato dextrose broth (PDB; Oxoid, Basingtoke, Hants, UK).

The same technique was used in dual cultures of *T. harzianum* and *F. oxysporum* f. sp. *gladioli*, the previous method was used, with addition of two 10 mm diameter plugs of *F. oxysporum* f. sp. *gladioli* into the same flask with *T. harzianum*. Cultures were incubated on rotary shaker at 220 rpm (Gallenkamp, Rhys Scientific Ltd, UK) for 15 days at 22° C.

#### Inoculation of Gladiolus corms

Gladiolus corms were inoculated with *T. harzianum* and *A. migulanus*, followed by inoculation with *F. oxysporum* f. sp. *gladioli*. The inoculated corms were incubated over moistened filter paper (Whatman No 1) with sterilized distilled water in plastic containers (22× 12× 32 cm) at 22° C. The filter papers were moistened daily under aseptic conditions.

#### Extraction and quantitation of secondary metabolites

The inoculated corms were frozen in liquid N and homogenized into fine powder using a hilled mortar and pestle. Aliquots (20 g) of the powder were extracted exhaustively with ethyl acetate (EtOAc) at room temperature for 3 times. The combined organic fraction was dried over  $\text{Na}_2\text{SO}_4$  and evaporated under reduced pressure at 35° C. The residue was taken up in 5 ml of EtOAc and subjected flash column

chromatography on a 600 x 30 mm column of Kieselgel 60 A, 0.40 – 63 micron (Merck, 50 g). The column was eluted in a EtOAc: hexane (1: 1 v/v) gradient. Fractions showing similar thin-layer chromatography (TLC) using aluminium backed Merck Kieselgel 60 F254 plates, 0.2 mm thickness. Fractions of 20 ml were collected and subjected to analytical TLC. Fractions showing similar TLC profiles based on UV detection at 254 nm were combined and purified compounds subjected to mass analysis using liquid chromatography-mass spectrometry (LC/MS; Thermo Instruments.) Further purification of the flash column chromatography residue from each treatment was carried out using high performance liquid chromatography (HPLC) on a C18 HD analytical column (250 mm x 4 mm; Agilent, USA), with an Agilent Series 1100 LC pump (Agilent, USA), coupled to an LC 90 UV spectrophotometer (Jasco International Co. Ltd.). Samples (100 µl) were eluted at room temperature in a linear gradient of 20 – 80% acetonitrile in acidified water (0.1% trifluoroacetic acid; Sigma- Aldrich) over 30 min. Purified compounds were characterized and identified using accurate mass analysis (LC/ MS). High resolution mass spectral data were obtained using a Thermo Instruments MS system (LTQ XL/ LTQ Orbitrap Discovery) coupled to a Thermo Instruments HPLC system (Accela PDA detector, Accela PDA autosampler and Accela Pump).

Compounds were identified, based on MS and NMR data by comparison with the Beilstein database (2010) and directly with mass and <sup>1</sup>H and <sup>13</sup>C NMR spectral data in the literature (Dunlop *et al.*, 1989). <sup>1</sup>H and <sup>13</sup>C NMR analysis were done on Varian 400MHz NMR spectrophotometer.

The filtered broth cultures of each treatment (1 L each); *T. harzianum* alone; *T. harzianum* + *F. oxysporum* f. sp. *gladioli* were extracted with ethyl

acetate (3 x 500 ml each). Cultures were filtered under vacuum through Whatman No. 4 filter paper (Brentford, UK) and the filtrates stored at 2° C for 24 h. Filtrates were extracted three times in 500 ml EtOAc, organic phases combined and rotary evaporated (BuchiRotavapor R-200, Switzerland) to dryness under reduced pressure at 40° C. The residue obtained from *T. harzianum* was brown (1.5 g); *T. harzianum* + *F. oxysporum* f. sp. *gladioli* reddish-brown (0.869 g), and *T. harzianum* + *F. oxysporum* f. sp. *gladioli* + *A. migulanus* yellow-brown (0.475 g). Residues were re-dissolved in 3 ml methanol each and further purified by flash column chromatography, using isocratic elution in EtOAc: hexane (2:1 v/v), as described above.

#### Biological activity of 6PP

The purified 6PP was tested against growth of *F. oxysporum* f. sp. *gladioli* using the method of Vinale *et al.* (2008). Discs of fungal mycelium on PDA, 10 mm in diam., were placed at the centre of 90 mm diam. Petri dishes containing fresh PDA. Test compounds were added in a 10 µl drop of methanol containing 10, 20, 50 and 100 µg of the compound per plug of *F. oxysporum* f. sp. *gladioli*. Cultures were incubated at 25° C for 7 days. Pathogen growth was measured daily. Each treatment consisted of three replicates and the experiment was repeated twice.

#### Effect of 6PP on Gladiolus growth in Hydroponic cultures

Gladiolus corms were grown hydroponically as described by Nosir *et al.*, 2009. The corms were treated with 10ppm solution of 6PP in Arabic gum and kept for drying at room temperature.

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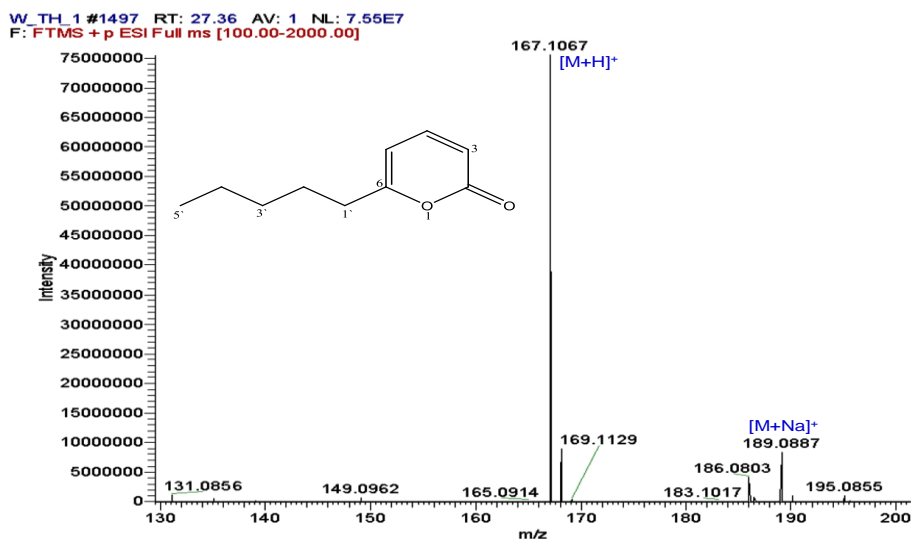


Fig. ( ) : Mass spectrum of 6PP

Figure 1: Mass spectrum of compound 1.

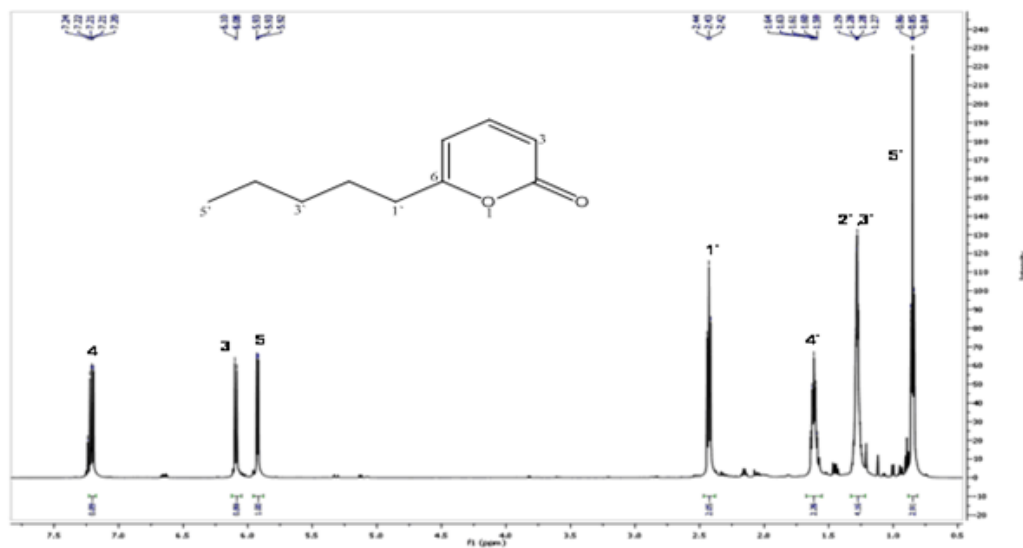


Figure 2: <sup>1</sup>H NMR spectrum of compound 1.



Figure 3:  $^{13}\text{C}$  NMR spectrum of compound 1.

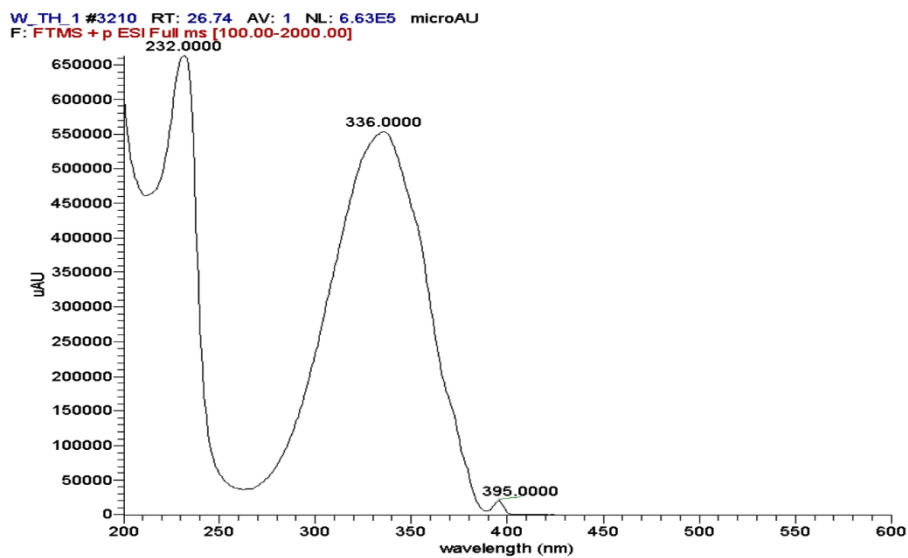


Figure 4: UV spectrum of compound 1.

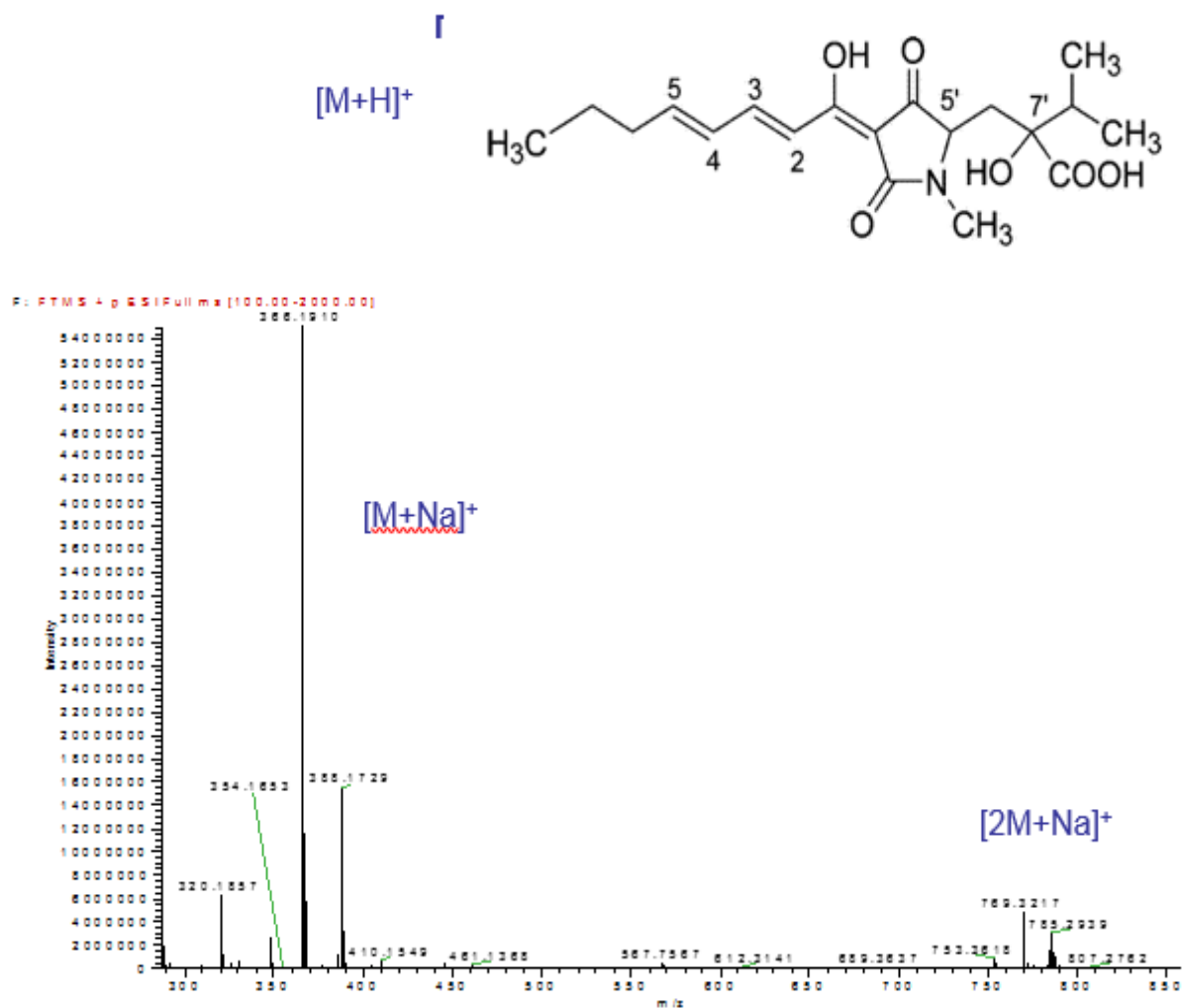
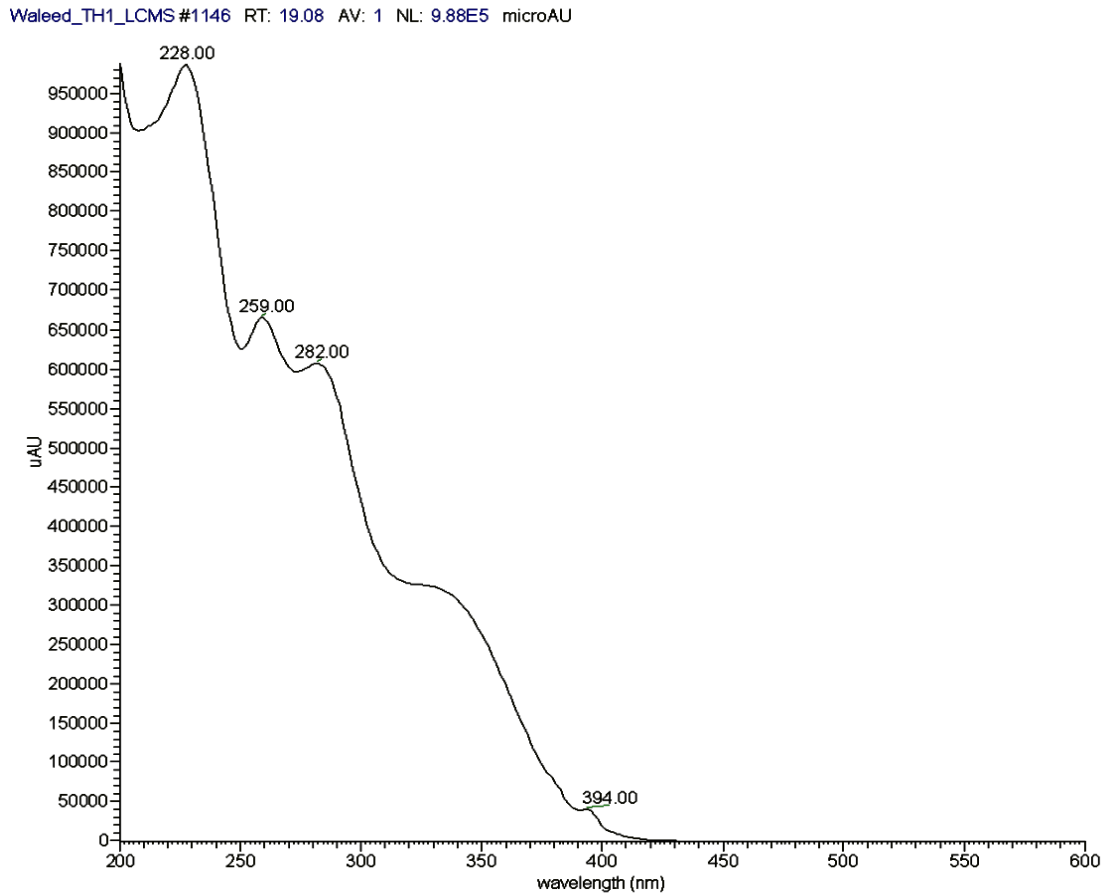


Figure5: Mass spectrum of compound 2.



**Figure 6: UV spectrum of compound 2.**

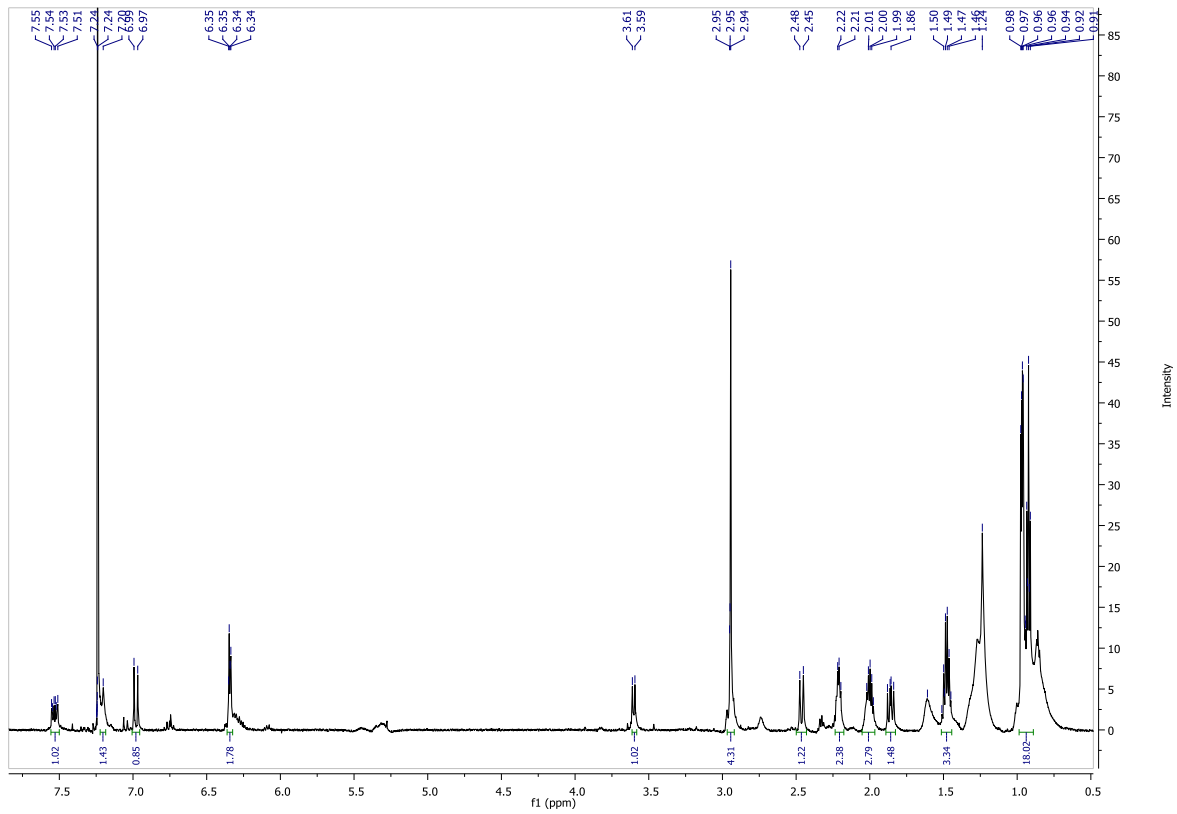


Figure 7: <sup>1</sup>H NMR spectrum of compound 2.

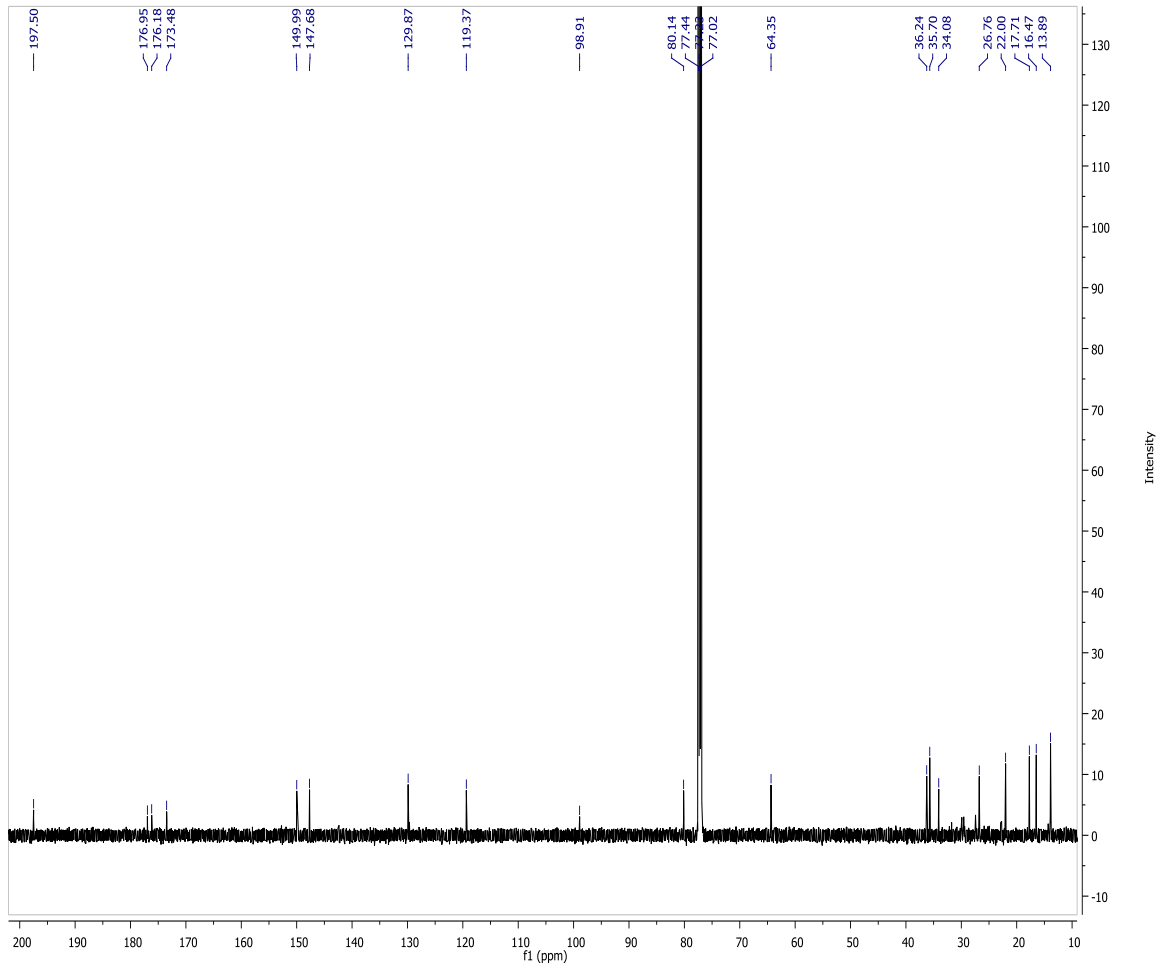


Figure 8:  $^{13}\text{C}$  NMR spectrum of compound 2.