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Fungal phytotoxins as natural herbicides for weeds biocontrol in pastures and crops

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ABSTRACT

A phytotoxic substituted salycilic aldehyde, named agropyrenol, produced in liquid culture by *Ascochyta agropyrina* var. *nana*, a potential mycoherbicide proposed for the control of perennial weed *Elytrigia repens*, was characterized together with other two new minor metabolites named agropyrenale and agropyrenone, respectively. When assayed on different weedy plants, agropyrenol showed a strong phytotoxic activity, agropyrenale was less active, while agropyrenone was inactive. Six derivatives obtained by chemical modifications of agropyrenol were assayed for phytotoxic, antimicrobial and zootoxic activities, and the structure-activity relationships were examined. Both the double bond and the diol system of the 3,4-dihydroxypentenyl side chain, as well as the aldehyde group at C-6 and C-1 of the phenolic ring of agropyrenol proved to be important for the phytotoxicity.

A new phytotoxic geranylcyclohexentriol, named phomentrioloxin, was isolated from the liquid culture of *Phomopsis* sp., a fungal pathogen proposed for the biological control of *Carthamus lanatus*, a widespread and troublesome thistle weed belonging to the Asteraceae family causing severe crop and pastures losses in Australia. Seven derivatives obtained by chemical modifications of the toxin were assayed for phytotoxic, antimicrobial and zootoxic activities, and the structure-activity relationships were examined. The hydroxy groups at C-2 and C-4 appeared to be important features for the phytotoxicity, as well as an unchanged cyclohexentriol ring. A role seemed also to be played by the unsaturations of the geranyl side chain.

These findings could be useful for understanding the mechanisms of action of novel natural products, for identifying the active sites, and possibly in devising new herbicides of natural origin.

1. INTRODUCTION

1.1. Weeds

The simplest definition of a weed is an “unwanted plant” referring to plants growing in habitats modified or managed by humans, and plants that cause damage to some segments of the human population (Norris, 1992). Thus, a further definition of a weed would be that “it is a plant growing at levels that interfere with management objectives in a given area and at a given point in time” (Ross & Lembi, 1985; Charudattan & DeLoach, 1988; Norris, 1992). But we can better define it with the definition of Kent (1994), which states: “a weed is a green plant that is growing in a location where its presence has become undesirable because of its adverse effects on the ecosystem or human activities”. The term weed applies to plants growing in habitats modified or managed by man, and refers to plants that some segment of the human population deems have an overall negative impact. The concept of a weed is site and time specific (Norris, 1992). A plant can be a weed in one situation but may not be classified as a weed in another (Kent, 1994), as weediness is not a fixed attribute of a plant (Norris, 1992).

Weeds have always been and will be an integral part of agricultural systems (Lovett & Knight, 1996). Smith (1983) reported that the most important plant families with the greatest number of species as weeds were Alismataceae, Poaceae, Asteraceae, Fabaceae, Lytheraceae and Scriphulariceae. Nearly 44 percent of the world’s worst weeds belong to family Poaceae, which happens to provide eight major crops, namely, wheat, maize, rice, sorghum, barley, millet, oat, and sugarcane (Kohli *et al*., 2006).

Perennial weeds are common problem in different crops. Weeds can compete with productive crops or pasture, or convert productive land into unusable scrub. They are also often poisonous, distasteful, produce burrs, thorns or other damaging body parts or otherwise

interfere with the use and management of desirable plants by contaminating harvests or excluding livestock.

Weeds tend to thrive at the expense of the more refined edible crops. They compete for light, nutrients, water and space (Glauninger & Holzner, 1982; Kropff, 1993). Without weed control, yield losses have been estimated to range from 16% to 86% or even 100% (Zoschke, 1990; Baltazar & DeDatta, 1992; Kropff, 1993). Losses are influenced by several factors which can be summarised as: (i) competitive efficiency of weeds, (ii) weed density, (iii) duration of competition, (iv) planting method, (v) crop species and variety, (vi) soil fertility level, (vii) water management, (viii) and interaction among the preceding factors (Chisaka, 1977; Smith, 1983).

Weeds assume large proportions of the area of their invasion. They possess certain traits or characteristics that make them ideal for proliferation. Traits such as the ability to reproduce at a faster rate, rapid growth from seedling to sexual phase, phenotypic plasticity, and high tolerance to environmental heterogeneity are associated with weedy plant species (Baker, 1974). Weed population are highly adaptable to production system through herbicidal resistance and shifts in their populations. Weeds possess adaptive strategies that determine their survival, growth and success in a particular environment (Holt, 1988). On the basis of intensity of stress and disturbance for successful establishment of a given area, plants can be stress-toleratant, competitors or ruderals (Grime, 1979). Weeds, however, falls into two combined categories: they can be competitive ruderals or stress-tolerant competitors (Grime, 1979). They take advantage of human-made habitats and are highly responsive to changes in environmental conditions in such a beneficial manner, enabling them to survive and grow in nature (Grime, 1979). Many annual, biennial and even perennial weed species found on arable fertile land are known as competitive ruderals that grow rapidly. Their competition occurs before flowering. Stress-tolerant competitors primarily trees or shrubs and even some perennial herbs, are characterized by rapid dry matter production, large stem extension, and

high leaf area production. In addition to grow strategies, weeds possess several other characteristics that make them successful colonizer of a given area (Kohli *et al*., 2006).

1.2. Weed management strategies

1.2.1 Traditional methods

A number of weed management strategies have been followed, but none probably provides a satisfactory solution to the weed problems. Broadly, four methods are employed for the management of weeds: mechanical, cultural, chemical and biological. Each of these methods has certain advantages and disadvantages. Among these, mechanical methods are one of the oldest, involving physical removals of weeds by soil disturbance prior to planting or by hand weeding or hoeing during crop growth. On the other hand, cultural methods are applied largely during the active growth period of the crop. This include crop rotation, use of cover, smother and green manure crops, crops residues, crop genotypes with better competitive and allelopathic ability and manipulation of sowing or planting date, crop density and crop pattern (Kohli *et al*., 2006). These are effective when they are able to enhance the differential development between crop and weeds to the advantage of the former (Mohler, 1996). These methods were in use in traditional agroecosystems, but with the modernization of agriculture and herbicide application these declined.

1.2.2 Chemical methods

The use of chemical methods is probably a twentieth-century technology when the herbicides, especially the hormonal ones discovered in 1940s, revolutionized agriculture. Their improved efficacy and production of herbicide-resistant crop have further expanded this revolution and become an important tool of modern weed management. They are widely used not only in the developed nations but also in developing nations such as India. A 350 percent increase in the use of herbicides occurred from 1971 to 1987 for control of weeds in rice and wheat fields in

India (Alstrom, 1990). Numerous herbicide families are known to have difference in unit activity, crop safety, toxicology and environmental effects. However, increasing herbicidal resistance, environmental and toxicological concerns have put a question mark on their large scale use (Burnside, 1993; Heap, 2005).

Combinations of mechanical, cultural, and chemical methods are more effective than any single method used alone (Trumble & Kok, 1982). Herbicides recommended for chemical control of the perennials in non-organic cropping systems are restricted to few active substances (clopyralid, dicamba, chlorsulfuron, bentazon, phenoxy-acids) (Lemna & Messersmith, 1990; Grekul *et al.*, 2005). These chemicals are frequently low specificity and are weakly biodegradable, accumulating in plants and in drinkable water, producing heavy environmental pollution, or creating problems to human and animal health (Evidente & Abouzeid 2006). During the last twenty years there has been mounting public concern about the effect of herbicides on both environment and public health. These include the effects of herbicides on surface and ground water, spray drift and long term impact of herbicide residues in agricultural products. This has led many governments to set specific targets to reduce the use of herbicides and the adoption of more sustainable crop production and protection methods by encouraging integrated pest management techniques (Evans et al., 1996; Lovett & Knights, 1996).

1.2.3 Biological methods

Management of weeds, should, therefore, be achieved through strategies that do not affect the sustainability of agroecosystems and the life support system.

Biological control was defined by Waterhouse (1978), as “the use of a pest’s natural enemies to keep its numbers below levels at which the damage caused becomes economically significant”. Biological control of weeds is a science that evolved in the twentieth century, with the first attempt at controlling weeds with insects in 1902 in Hawaii and a few years later

in Australia (Bruzzese, 1990). This method of control has advantages over mechanical and chemical control methods, especially chemical control which is a temporary, expensive method, that is not always applicable (Hassan, 1980).

Unlike chemical compounds, biological control agents can be specific to a particular weed and cause no environmental pollution problems (Hassan, 1980; Templeton, 1985), often with high specificity and represent a long term solution also in the control of weed particularly resistant to chemical herbicides. The application of biological weed control offers significant opportunities not only for farmers, nature conservationists and other vegetation managers but also for institutions and companies that wish to sell plant protection services and products, and for the general public that demands safe food and a visually attractive and different environment.

1.2.4 Phytopathogenic fungi

New herbicides have become of great interest due to either the few natural product derived commercial herbicides already in use or the rapidly evolving resistance to current herbicides. This supports the need for more effort to be expended on a natural product derived herbicides and makes attractive the prospect of evaluating a vast number of undiscovered or understudied natural compounds that are likely to have biological activity (Evidente et al., 2011a; Evidente and Abouzeid, 2006). The identification and the biological and molecular characterization of microorganisms, useful as biocontrol agents or as producers of bioactive compounds, are of great interest for the modern and echo-friendly agriculture. Among the microorganisms, fungi are the most common pathogens of plants and therefore for weeds as well. Some insects and fungi, which satisfy the criteria of efficacy, specificity and long-time persistence, have been already commercialised essentially outside from Europe (Bottiglieri et al., 2000). The use of phytopathogenic fungi in biological control of weeds may represent a promising alternative to the use of chemicals or in Integrated Weed Management Systems

(IWMS). Researches in this field are carried out according to the two fundamental strategies: the inundative and the classical method. The first one consists in the application of the pathogenic agent in the environment, as for herbicides, so that these pathogens are usually called "mycoherbicides". With the classical approach the biocontrol agent is introduced in a restricted infested area and, subsequently, allowed to spread. An alternative approach to weed biocontrol is the use of toxic metabolites produced by weed pathogens, in addition or in alternative to the pathogen, or in integrated weed control programs. The replacement or the integration of traditional chemical control methods to plant disease by the use of microorganisms and/or their bioactive metabolites reduces the environmental impact of agricultural productions and gives efforts to the agricultural biological production which is more and more present in the national and international markets. These bioactive secondary metabolites could play an interesting role in the induction of disease symptoms (phytotoxins) or of defence response (elicitors).

The first approach is the isolation of microorganisms from tissues of infected infesting plants, followed by selection of the strains with higher specificity and virulence. The second step is to find appropriate conditions for the *in vitro* growth of the fungi to obtain culture filtrates with high phytotoxicity against the host plants. Next, the phytotoxins are isolated, characterized and in some cases derivatized before to be tested as potential herbicides.

Finally, the knowledge of the chemical structure of these substances may allow the partial or total synthesis of the most appropriate natural herbicide. Furthermore, (if they are a virulent factor), the toxins could be used in indirect mode as biomarkers, to select the best fungal strain or to optimise for their large scale production (Evidente, 2006; Evidente and Abouzeid 2006, Evidente et al, 2011a) and in combination with low dose of herbicides and the phytopathogenic fungus, to develop Integrated Weed Management Strategy.

1.2.5 Integrated Weed Management Systems

IWMS is usually considered as part of Integrated Pest Management Systems (IPMS) that is an approach in which principles, practices, methods, materials and strategies are chosen to control pests while minimising undesirable results (Shaw, 1982; Smith 1991). IWMS is a systems approach (Swanton & Murphy, 1996, encompassing different methods of weed management together with effective education and extension of the management components that takes into account the whole range of issues from agricultural production, to economic losses, risks to human health and the environment and energy requirement (Shaw, 1982; Blair & Parochetti, 1982; Lovett & Knights, 1996). The objectives of IWMS are the reduction of losses caused by weeds, costs of control, energy and labor requirements, assure adequate supply of food, improve environmental quality and maximise producer profits (Penner, 1982; Shaw, 1982; Fischer et al., 1993).

IWMS combines the use of several methods (Chisaka, 1977; McWhorter & Shaw, 982; Smith, 1991; Lovett & Knights, 1996) which can be summarised as:

1) ecological methods that include multiple-pest-resistant, high yielding, well-adapted crop cultivars that resist weed competition, fertiliser management to give the crop competitive advantage, careful crop rotation, optimum crop plant population and use of allelopathy in crop plants to interfere with weeds. Such methods also include the use of judicious irrigation practices;
2) physical methods include appropriate cultivation, field sanitation and harvesting methods that do not spread weed seeds, appropriate seedbed tillage and seeding methods that enhance crop growth while minimising weed growth. Minimum tillage, direct drilling and zero tillage to reduce disturbance of the soil systems which can be enhanced by chemical methods also fall into this category of control method;

3) chemical methods of weed management include the use of herbicides and genetically engineered herbicide-resistant crops. However, it is important that herbicide-resistant crops are not promoted as a panacea, but as a component of IWMS;
4) biological methods usually include the use of organisms such as fungal or bacterial pathogens, insects, plants and herbivores. The use of bioherbicides may be the best choice of biological control method in cultivated crops.

1.2.6 Fungal phytotoxins

Plant pathogens are good sources of potent phytotoxins (Abbas & Duke, 1995; Evidente & Motta 2001), as they usually kill tissues before they consume them. In spite of this, the fungi that kills weed species have received relatively little attention in natural product herbicide discovery efforts, with a notable exception. Maculosin, produced by *Alternaria alternata* (Fr.) Keissl., a pathogen responsible for diseased spotted knapweed (*Centaurea maculosa* Lam.), is the first phytotoxins with a high degree of host specificity. Moreover, other studies on the biological activity of this phytotoxin and its practical application as a knapweed control agent were described in previous work by Strobel (1991).

Almost all fungal species produce phytotoxic metabolites (Evidente & Motta, 2001; Evidente & Abouzeid 2006). Phytotoxins are defined as microbial metabolites that are harmful to plants at very low concentrations. Most of the plant pathogenic fungi produce toxins in *in vitro* cultures and on *in vivo* hosts. Frequently, these compounds play an important role in the pathogenesis as reproduce some or even all of the symptoms of the disease. In many cases the toxins are low molecular weight compounds belonging to a variety of class of natural products. They are able to diffuse from the site of the infection to surrounding tissues or to move within the plant. The virulence of the plant pathogens may depend on their capability to synthesize one or more toxins.

Fungal phytotoxins have facilitated advances in our understanding of various phenomena in plant and fungal physiology, biochemistry, genetics, and molecular biology. During the past few decades, phytotoxins have been employed as tools contributing to fundamental discoveries in plant pathogenesis, host specificity, mechanisms of resistance and susceptibility, secondary metabolism, fungal genome organization, plant cell and organelle functions, and fungal ecology (Ballio & Graniti, 1991).

Fungal phytotoxins can be classified as host-selective or non-selective. Although it is difficult to find a clear line of demarcation, host selective toxin are usually highly toxic only to host-species or cultivars susceptible to the producing pathogen. Non host or resistant cultivars are less sensitive to these toxins (Graniti et al., 1989).

It is possible to isolate phytotoxins from infected plant tissues and germinating conidia of fungi, but this approach in not productive because of the low content of the target compounds. Therefore, in order to isolate phytotoxins in amounts sufficient for studies of chemical and biological properties, the fungi are cultured in liquid nutrient media (the average yield ranges from 1 to 50 mg per 1 liter of liquid culture). In a number of cases, it is possible to isolate phytotoxins in settings that involve solid phase fermentation on natural substrates (Berestetskiy, 2008).

Phytotoxins production is sensitive to a number of different factors (e.g., the composition of the medium, its acidity, and the duration and conditions of culturing), most of which are not identified in advance as being able to affect the process. Distinct strains of the same species may be very considerably in their capacity for phytotoxins production (Berestetskiy, 2008). Microorganism strains are genetically unstable, and their storage or reinoculation may adversely affect the ability to produce toxins (Kale end Bennet, 1992).

Phytotoxins produced by fungal pathogens cause necrotic symptoms in most cases. It has been long assumed that such pathogens kill host tissue by producing the toxin in advance of the colonization and live as saprophytes from the dead substrate. Actually, leakage of cell

constituents frequently occurs after the application of both host-specific and non-host-specific phytotoxins.

Many fungal genera are well known for the ability to produce metabolites with biological activities, in which among them, the phytotoxic activity. Some of these fungi have been proposed for mycoherbicides for the biocontrol of weeds species such as: *Cirsium arvense* (L.) Scop., *Chenopodium album* L., grass weeds, *Sonchus arvensis* L. etc. (Fig. 1). Their pathogens, belonging to genuses as *Alternaria, Ascochyta, Drechslera, Phoma, Phyllostictica, Pyrenophora. Septoria, Stagonospora,* showed to produce *in vitro* many metabolites belonging of different classes of natural compounds as reported below.

1.2.6.1 *Chenopodium album*

Chenopodium album is a common worldwide weed of arable crops such as sugar beet and maize. *Ascochyta caulina* (P. Karst.) v.d. and v. Kest and *Phoma chenopodiicola* Gruyter, Noord. & Boerema were proposed as mycoherbicide for its biological control.

A. caulina, is a promising biological control agent for the control of *C. album*, known as common lambsquarter or fat hen, which is a common weed of arable crops such beet and maize world-wide. *A. caulina* produce in liquid culture, low molecular weight hydrophilic phytotoxins named ascaulitoxin, its aglycone and 4-aminoproline (Fig. 2). These toxins have been chemically characterized by spectroscopic and chemical methods as the β-D-glucopyranoside of 2,4,7-triamino-5-hydroxyoctandioc acid, the acid itself, and *trans*-4-amino-D-proline, which are all non-protein amino acids and their stereostructures were also determined (Evidente and Abouzeid, 2006).

When assayed on punctured leaves of host and non host plants, including wild and cultivated, the three metabolites showed very high phytotoxicity on *C. album* and also on other very noxious weeds and cultivated plants, with only very weak activity observed on some agrarian crops. Unlike ascaulitoxin and *trans*-4-amino-D-proline appeared to have greater phytotoxic

specificity towards dicots, they were nontoxic to several monocots (Evidente and Abouzeid, 2006).

These results, together with observed lack of activity against fungi and bacteria, lack of toxicity to brine shrimp larvae (*Artemia salina* L.), and high water solubility make these amino acids good lead compounds for development as safe natural herbicides (Evidente and Abouzeid, 2006).

In order to establish their mode of action, considering their amino-acid nature, it was evaluated the ability of ascaulitoxin and its aglycone to interfere on amino acid metabolism on *Lemna paucicostata* H. These studies allowed to formulate three possible hypothesis: 1) the toxin inhibits one or more aminotransferases not examined, 2) ascaulitoxin aglycone affects amino acid transporters, 3) ascaulitoxin aglycone is a protoxin that is converted *in vivo* to an aminotransferase inhibitor (Duke et al., 2011).

Also, recently a research project "ECOVIA-Development and pre-industrialization of an environmentally sound natural herbicide", granted from the Lombardy (Italy) Regional Governorate, allowed to carry out studies aimed to obtain a natural and safety herbicide based on the bioactive metabolites produced by *A. caulina* and to investigate on their toxicity.

Furthermore, a simplified method of scaling up of the whole process, including a single-step purification of the fungal culture filtrates by cationic exchange chromatography by using a reusable resin with a high exchange capacity, with distilled water and dilute ammonia as eluents, was optimized. This allowed the fast and easy purification of large volumes of fungal culture filtrates and the harvest of large amount of a mixture containing the three pure toxins for practical purposes (Avolio et al., 2011; Vurro et al., 2012).

Finally, ecotoxicologic studies were carried out on mixture of *A. caulina* toxins (ACTs). The studies provided baseline information needed to assess the potential hazard of the fungal toxins of *A. caulina*. In particular, the acute and chronic effects on aquatic (algae, *Daphnia* and fish) and terrestrial organisms (earthworms) was investigated. In particular no toxic

effects were observed on target organisms (Fumagalli et al., 2013). A comparison of the inherent toxicity of ACTs with other synthetic herbicides showed comparative ecotoxicity of the tested mixture (Fumagalli et al., 2013).

A phytotoxic unrearranged ent-pimaradiene diterpene, named chenopodoline, was more recently isolated from the liquid culture of *P. chenopodiicola.* Its structure was established by spectroscopic, X-ray, and chemical and optical methods 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 (Fig. 3). The toxin caused necrotic lesions on different weeds (Cimmino et al., 2013a). Successively, three tetrasubstituted furopyrans, named chenopodolans A–C, and (-)-(*R*)-6-hydroxymellein were isolated from the same culture filtrate. The structures of chenopodolans A–C were established by spectroscopic and chemical methods as 2-(3-methoxy-2,6-dimethyl-7aH-furo[2,3-b]pyran-4-yl)-butane-2,3-diol, 1-(3-methoxy-2,6- dimethyl-7aH-furo[2,3-b]pyran-4-yl)ethanol and 3-methoxy-2,6-dimethyl-4-(1-methylpropenyl)-7aHfuro[2,3-b]pyran, respectively (Fig. 3). 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 (Ohtani et al. 1991). Assayed by leaf puncture on host and non-host weeds, chenopodolans A and B and the 11-*O*-acetylchenopodolan A showed a strong phytotoxicity (Cimmino et al. 2013b).

1.2.6.2 *Cirsium arvense*

C. arvense (common name: canada thistle) is a persistent perennial weed that grows vigorously, forming dense colonies and spreading by roots. It spreads by seed, either by wind or as a contaminant in crop seed. Canada thistle is native to south eastern Europe and the eastern Mediterranean area. It has spread to most temperate parts of the world and is considered an important weed all around the world as it infests many habitats such as cultivated fields, roadsides, pastures and rangeland, railway embankments, and lawns (Holm et al., 1977).

Phyllosticta cirsii Desm., a fungal pathogen isolated from *C. arvense* (Canada thistle) and proposed as biocontrol agent of this noxious perennial weed, produces in liquid cultures different phytotoxic metabolites with potential herbicidal activity. Four new oxazatricycloalkenones, named phyllostictines A-D (Fig. 4), were isolated and characterized using essentially spectroscopic and chemical methods. Tested by leaf-puncture assay on the fungal host plant phyllostictine A proved to be highly toxic (Evidente et al., 2008a). The phytotoxicity decreases when both the dimension and the conformational freedom of the macrocyclic ring change, as in phyllostictines B and D, and it is totally lost when also the functionalization of the same ring is modified, as in phyllostictine C (Evidente et al., 2008a).

Further purification of the same organic extract provided two other metabolites, named phyllostoxin and phyllostin (Fig. 4), which were characterized by spectroscopic technique (essentially NMR and MS). Phyllostoxin and phyllostin proved to be a new pentasubstituted bicyclo-octatrienyl acetic acid ester and a new pentasubstituted hexahydrobenzodioxine carboxylic acid methyl ester, respectively. When tested on punctured *C. arvense* leaves, phyllostoxin proved to be highly phytotoxic, causing rapid and large necrosis, whereas phyllostin had no phytotoxicity in this bioassay (Evidente et al., 2008b).

Stagonospora cirsii Davis, a fungi proposed as a potential mycoherbicide of Canada thistle produced phytotoxic metabolites in liquid and solid cultures. From the liquid culture, the main metabolite, named stagonolide (Fig. 5), and characterized as a new nonenolide, was isolated. Its biological activities were also extensively studied (Yuzikhin et al., 2007). When the same fungus was grown in solid culture, exhibited an increased capacity to produce nonenolides. Five new nonenolides, named stagonolides B-F (Fig. 5), were isolated and characterized using spectroscopic methods. When tested by a leaf disk puncture assay, these compounds showed no toxicity to host plant (Evidente et al., 2008c).

A further four nonenolides were isolated and characterized by spectroscopy. Three were new compounds and named stagonolides G-I, and the fourth was identified as modiolide A,

previously isolated from *Paraphaeosphaeria* sp. Verkley & Aa (Fig. 5). On leaf disk-puncture assays, stagonolides H-I and modiolide A were phytotoxic to *C. arvense*. Only stagonolide H inhibited chicory seedling root growth. The most potent toxin, stagonolide H, showed selectivity when tested on leaves of eight different plants: Canada thistle was the most sensitive to the compound (Evidente et al., 2008d).

1.2.6.3 Grass weeds

In many countries, annual and perennial grasses are among the most problematic weeds for various crops (Holm et al., 1977). Such weeds are difficult to control because of their prodigious seed production, which is responsible for their reproduction and diffusion, their tolerance to the chemical herbicides available, and their growth habits that can enable them to escape from chemical and mechanical control practices. Considering the increasing number of weed species that are tolerant or resistant to the use of herbicides (Naylor, 2002), and the difficulties in finding new chemical active compounds, biocontrol microorganisms and new herbicides from natural sources are receiving a renewed interest. Pathogenic fungi isolated from grass weeds were found in several fungal collections and many strains were collected (Fracchiolla, 2003). Such investigation was aimed at finding producers of toxic metabolites with herbicidal activities against grass weeds. Some of the selected fungal strains were able to produce highly phytotoxic culture filtrates.

Drechslera gigantean (Heald & F.A. Wolf) S. Ito, a potential mycoherbicide of grass weeds, was isolated in Florida from naturally infected large crabgrass (*Digitaria sanguinalis* (L.) Scop.). It produces phytotoxic metabolites when grown in liquid culture. The main metabolite, identified by spectroscopic and optical methods was identified as ophiobolin A (Fig. 6), a well-known phytotoxic sesterterpene produced by several phytopathogenic fungi of important crops and already extensively studied for its interesting biological activities (Evidente et al. 2006a). Successively, other seven minor metabolites related to ophiobolin A,

were isolated using the same techniques: 6-epi- and 3-anhydro-6-epi-ophiobolin A, ophiobolin B, E, I, J and 8-*epi*-ophiobolin J (Fig. 6). Assayed on punctured detached leaves of several grass and dicotyledon weeds, ophiobolin A proved to be on average more phytotoxic as compared to the other related compounds (Evidente et al. 2006b).

Pyrenophora semeniperda (Brittleb. & D.B. Adam) Shoemaker (anamorph: *Drechslera*) is a seed pathogen proposed for cheatgrass (*Bromus tectorum* L.) (Fig. 1) biocontrol. It showed to produce in solid and liquid cultures metabolites belong to different class of natural compounds.

Three cytochalasans, named cytochalasins Z1, Z2 and Z3 (Fig. 7), were isolated from the wheat culture of fungus, with cytochalasins F, deoxaphomin and cytochalasins B (Fig. 7). Cytochalasins Z1 and Z2 were structurally related to cytochalasin T, whereas cytochalasin Z3 was related to cytochalasin B. When assayed on wheat and tomato seedlings, cytochalasin Z3, in comparison to cytochalasin B, its 21,22-dihydroderivative, cytochalasin F and deoxaphomin showed a remarkable ability to inhibit root elongation (Evidente et al. 2002).

More recently, from the liquid cultures of a strain of *P. semeniperda*, isolated from *B. tectorum*, in addition to cytochalasin B, a new spirocyclic γ-lactam, named spirostaphylotrichin W, was isolated together with the well known and closely related spirostaphylotrichins A, C, D, R and V, as well as triticone E, (Fig. 8). Spirostaphylotrichin W was characterized as (3*S**,4*S**,5*S**,6*S**,9*Z*,10*Z*)-4,6- dihydroxy-2,3-dimethoxy-3-methyl-10-propyliden-2-azaspiro [4.5]dec-8-ene-1,7-dione, by spectroscopic and chemical methods (Masi et al., 2014). In a *B. tectorum* coleoptile bioassay at concentration of 10^{-3} M, spirostaphylotrichin A proved to be the most active compound, followed by spirostaphylotrichins C and D. Spirostaphylotrichin W and V showed mild toxicity while spirostaphylotrichin R and triticone E were not active. When tested on host and non-host plants by leaf puncture bioassay, spirostaphylotrichins A, C and D caused the appearance of necrotic spots while the other compounds were inactive (Masi et al., 2014).

1.2.6.4 *Sonchus arvensis*

Classified as a noxious weed in many states and provinces, *S. arvensis* (common name: perennial sowthistle) is a problem in several crops, where it causes economic losses due to reduced crop yields, increased cultivation and herbicide expenses, and land depreciation. Native of Eurasia, perennial sowthistle is distributed from South Scandinavia to Italy and from east to the western portions of the former Soviet Union (Holm et al., 1977). Since its introduction to North America, it has spread widely throughout the northern United States and southern Canada. The plant has also established in South America, Australia, and New Zealand. Widely established in temperate regions, it is not found in the tropics (Lemna and Messersmith 1990).

Alternaria sonchi Davis is a fungal pathogen isolated from *S. arvensis* and proposed as a biocontrol agent of this noxious perennial weed. Different phytotoxic metabolites were isolated from solid culture of this fungus. Two new polycyclic ethanones, named alternethanoxins A and B, were isolated and characterized using essentially spectroscopic and chemical methods (Fig. 9). Tested by leaf disk-puncture assay on the fungal host plant and a number of nonhost plants, alternethanoxins A and B showed to be phytotoxic, whereas they did not possess antimicrobial activity.

Hence, alternethanoxins A and B have potential as nonselective natural herbicides (Evidente et al., 2009).

Phoma exigua var. *exigua* Desm., a fungal pathogen isolated from *C. arvense* and *S. arvensis*, proposed as a biocontrol agent of these two weeds, produces in liquid and solid cultures different phytotoxic metabolites with potential herbicidal activity. The phytotoxic cytochalasins B, F, Z2, and Z3 and deoxaphomin (Fig. 7) were identified together with *p*-hydroxybenzaldehyde (Cimmino et al., 2008). Using spectroscopic methods, four cytochalasins, termed phomachalasins A-D (Fig. 10), were isolated and characterized as three new closely related 26-oxa[16] and one new [15]cytochalasans. They appeared to belong to a

new subgroup of cytochalasans bearing a 1,2,3,4,6,7-hexasubstituted bicycle[3.2.0]heptene joined to the macrocyclic ring (Evidente et al., 2011b).

None of the four new cytochalasans showed phytotoxic or antimicrobial activity. The lack of both phytotoxic and antimicrobial activities showed by all phomachalasins A-D was probably due to the strong modification of both functionalities and conformational freedom of the macrocyclic ring caused by its junction with the bulky and quite rigid new bicycle, namely bicycle[3.2.0]heptene (Evidente et al., 2011b).

1.3 *Elytrigia repens*

Elytrigia repens (L.) Desv. ex Nevski is a perennial weed widespread in Europe, Asia and north-west America. It could be from 40 to 150 cm high and it has a straight and rough stem (Fig. X). The blue-green coloured leaves, are linear, thin and flat and could present upfront short hairs. The green and long ears are made sessile spikelets and the green flowers, from 4 to 6 each spikelet. They are enclosed in two glumes and two glumella and they have three stamens (Fig. 11). The plant reproduces via semen or vegetatively with a “creeping” external rhizome provided with rootlets on the nodes.

This species is able to emerge all year long and it is easy to find it on the streets, on river’s banks, in uncultivated fields, from plains to mountain regions up to 2000 meters. It is able to destroy all the cultures (i.e. corn, barley, wheat, linen, oats, rye, sugar beet, soy and sunflower) with a strong competition (Evidente *et al.*, 2009).

For the biological control of *E. repens* biocontrol strategies, *Ascochyta agropyrina* (Fair.) Trotter var. *nana* resulted particularly interesting as mycoherbicide. This fungus belong to a very well known genus able to produce bioactive secondary metabolites. That is why a study on the isolation of bioactive metabolites from this fungus grown on both solid and liquid culture was undertaken.

In a previous paper (Evidente *et al.*, 2009) papyracillic acid was isolated for the first time from the fungal solid culture of *A. agropyrina* var. *nana* (Fig. 12). It was identified as a new 1,6-Dioxaspiro[4,4]nonene using spectroscopic methods, including X-ray diffractometric and CD analysis for the assignment of the relative and absolute stereochemistry. Some key derivatives were prepared and used in a structure-activity relationship study. Tested by leaf disk-puncture assay, papyracillic acid showed to be phytotoxic both for the host plant and a number of nonhost plants of the fungus. Papyracillic acid was active against bacteria (*Xanthomonas campestris* (Dowson) Dye and *Bacillus subtilis* (Ehrenb.) Cohn) and the fungus *Candida tropicalis* (Castell.) Berkhout. Some key derivatives were also prepared and used in structure-activity relationship study. Tested on puncture leaf bioassays, papyracillic acid showed to be phytotoxic for both host and non-host plants. Derivatives of papyracillic acid were significantly less active than parent toxin.

1.4 *Carthamus lanatus*

Carthamus lanatus L. (common name: saffron thistle), native of the Mediterranean region, is a widespread and troublesome weed of cropping and pastoral areas of Australia. Introduced to Australia in the period 1860-70 (Watson, 1990), it has spread through most of the southern half of Queensland, and occurs in New South Wales, Victoria, Tasmania, South Australia and Western Australia (Friend, 1983). Today it is regarded by New South Wales Agriculture as the most widespread thistle in pastoral areas and the third most important weed of pastoral land in New South Wales (Fig. 13). In cereal crops it decreases yield and results in dockage through contamination of grain (Pierce & Quinlivan, 1968). In pastures it prevents access and reduces carrying capacity (Meadley, 1957).

Saffron thistle is described as a winter growing annual with a rosette of stiff leaves at the base and an upright flowering stalk of up to 1 meter high (Meadley, 1957). Basal leaves are petiolate, deeply lobed, each ending in a spine. The main stem is very branched at the top,

leaves are sessile, partially stem-clasping, prominently veined, lobed and spiny. The main stem and branches end in solitary, yellow flower heads, wach surrounded by stiff, spiny, leaf-like, hairy bracts (Fig. 14) (Auld & Medd, 1987).

In Australia a certain number of pathogenic isolates of *Phomopsis sp.* was isolated from naturally infected plants and their potential mycoherbicidal activity was evaluated. A study on the genetic biodiversity carried out on a wide number of fungal strains ascertained that *Phomopsis* sp., saffron thistle's pathogens, is a tipical Australian species (Ash et al., 2010). *In vitro*, the fungus caused a quick appearance of the symptom may be inducted from the phytotoxins produced by the fungus itself during the disease's process of development. Recently, the teleomorph form of this pathogen was classified as *Diaporthe gulyae* R.G. Shivas, S.M. Thompson & A.J. Young *sp. nov.* The identification of *D. gulyae*, associated with stem canker of sunflower (Helianthus annuus) in Australia was studied using morphology, DNA sequence analysis and pathology. Over 300 isolates of *Diaporthe* (*Phomopsis*) were obtained from stems, leaves and seed of both cultivated and wild sunflower plants exhibiting symptoms of stem canker across New South Wales and Queensland. Phylogenetic analysis revealed that *D. gulyae* not correspond with known taxa, and this is believed to represent a novel species. (Fig. 15) (Thompson et al., 2011).

2. OBJECTIVES

The aim of the present thesis was the study of the phytotoxins produced by *Ascochyta agropyrina*, a fungal pathogen isolated from *Eletrygia repens*, and those produced by *Diaporthe gulyae*, a fungal pathogen isolated from *Carthamus lanatus*, and proposed as biocontrol agent of their respective noxious perennial weed. The chemical and biological characterization of the phytotoxins was carried out in order to understand the role of these natural products in the pathogenesis process and therefore to use them against specific diseases. The steps proposed to achieve this goal are summarized in the following points:

- Isolation and chemical characterization, by spectroscopic technique and chemical methods, of the phytotoxic metabolites produced *in vitro* by *Ascochyta agropyrina*, a potential mycoherbicide for *Eletrygia repens* biocontrol.
- Isolation and chemical characterization, by spectroscopic technique and chemical methods, of the phytotoxic metabolites produced *in vitro* by *Diaporthe gulyae*, a potential mycoherbicide for *Carthamus lanatus* biocontrol.
- Hemisynthesis of agropyrenol derivatives to use them in a structure-activity relationship study for the evaluation of their phytotoxic, antifungal, antimicrobial and zootoxic activity.
- Hemisynthesis of phomentrioloxin derivatives to use them in a structure-activity relationship study for the evaluation of their phytotoxic, antifungal, antimicrobial and zootoxic activity.

3. MATERIALS AND METHODS

3.1. Fungi

Ascochyta agropyrina var. *nana* Punith was isolated and identified from naturally infected leaves of *Elytrigia repens* (commonly known as quack grass). A monoconidial isolate was deposited in the culture collection of both the All-Russian Research Institute of Plant Protection, Pushkin, Saint-Petersburg, Russia (A-10) and the Institute of Science of Food Production, Bari, Italy (ITEM 12530). The isolate was routinely grown and maintained in plates and slants containing PDA (potato-dextrose agar).

The fungal strain of *Diaporthe gulyae* used in this study was isolated from symptomatic saffron thistle (*Carthamus lanatus*) plants in Australia in 1994 (Crump et al. 1996). The fungus was identified by Dr Micheal Priest (New South Wales Department of Primary Industries, ASCU Orange Agricultural Orange, Australia) and deposited as DAR Herb 73822 saffron thistle (*C. lanatus*) plants in Australia in 1994 (Crump et al. 1996). Pure cultures were maintained on potato-dextrose-agar (PDA, Sigma-Aldrich Chemic GmbH, Buchs, Switzerland) and stored in the collection of the "Istituto di Scienze delle Produzioni Alimentari, CNR, Italy," with the code ITEM13496.

3.2. General Procedures

Optical rotation was measured in $CHCl_3$, unless otherwise noted, solution on a Jasco P-1010 digital polarimeter; IR spectra were recorded as glassy film on a Perkin-Elmer Spectrum One FT-IR Spectrometer and UV spectra were taken in MeCN solution on a Perkin-Elmer Lambda 25 UV/Vis spectrophotometer. ^{1}H and ^{13}C NMR spectra were recorded at 600/125 MHz and at 400/100 MHz in $CDCl_3$ on, unless otherwise noted, 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 ESIMS 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 (Merck, Kieselgel 60 F_{254}, 0.25 and 0.50 mm, respectively) or KC18 F_{254}, 0.20 mm reverse phase (Whatman) plates; the spots were visualized by exposure to UV light and/or by spraying first with 10% H_2SO_4 in methanol and then with 5% phosphomolybdic acid in ethanol, followed by heating at 110 °C for 10 min. Column chromatography (CC) were performed on silica gel (Merck, Kieselgel 60, 0.063-0.200 mm). X-ray data collection was performed at 173 K under N_2 flow on a Bruker-Nonius KappaCCD diffractometer equipped with a graphite monochromated MoKα radiation (λ= 0.71073 Å, CCD rotation images, thick slices, φ and ω scans to fill asymmetric unit).

4. EXPERIMENTAL

4.1. Production, extraction and purification of phytotoxins from *Ascochyta agropyrina* var. *nana* culture filtrates

The fungus *Ascochyta agropyrina* var. *nana* was grown on a mineral defined liquid media named M1-D (Pinkerton and Strobel, 1976). Roux bottles (1 L) containing M1-D (200 ml) were seeded with mycelial fragments obtained from colonies actively growing on PDA plates. The cultures were incubated under static conditions at 25 °C in the dark for four weeks, then filtered on filter paper (Whatman n. 4), assayed for phytotoxic activity and lyophilised. The culture filtrate (8.6 L), showing high phytotoxic activity on punctured leaves, was lyophilized, dissolved in distilled water (1/10 of its original volume, pH 4) and then extracted with $CHCl_3$ (4 x 860 ml). The organic extracts were combined, dehydrated with Na_2SO_4 and the solvent evaporated under reduced pressure, yielding a brown oil (1.872 g). All the successive steps of the purification were guided by the phytotoxic assay on punctured leaves. The extract was purified by CC with $CHCl_3$-*i*-PrOH (95:5), yielding 10 groups of homogeneous fractions (1-10) (Scheme 1). Fraction 7 (399.0 mg) was purified by column chromatography with $CHCl_3$-*i*-PrOH (9:1), yielding 8 groups of homogeneous fractions (A-H). Fraction E (199 mg) was further purified by preparative TLC with $CHCl_3$-*i*-PrOH (95:5), yielding 4 groups of homogeneous fractions (E1-E4). E2 (159.4 mg) was finally purified by TLC on reverse phase with H_2O-EtOH (6:4), giving a yellow amorphous pure solid named agropyrenol (**1**, 125.9 mg, 14.6 mg/l, R_f 0.36). Fractions 4 and 5 of the original column were combined (219 mg) and purified by CC with EtOAc-*n*-hexane (6:4), yielding 9 groups of homogeneous fractions (A-I). The first three fractions (A-C) were combined (40.3 mg) and further purified by TLC with $CHCl_3$-*i*-PrOH (95:5), giving two amorphous solids named agropyrenale (**2**, 2 mg, 0.23 mg/l, R_f 0.41) and agropyrenone (**3**, 24.1 mg, 2.8 mg/l, R_f 0.30), respectively.

4.1.1. Agropyrenol (1)

Agropyrenol (**1**), obtained as yellow amorphous solid had: $[\alpha]^{25}_{D}$ -47 (*c* 0.5, MeOH); IR ν_{max} 3369, 2852, 1634, 1607, 1571, 1449 cm^{-1}; UV λ_{max} nm (log ε) 351 (3.49), 276 (3.83), 230 (4.22); ^{1}H and ^{13}C NMR spectra: see Table 1; ESIMS (+) *m/z* 245.081 [calcd. for $C_{12}H_{14}NaO_4$ 245.0790, M + Na]$^+$

4.1.2. Agropyrenale (2)

Agropyrenale (**2**), obtained as amorphous solid had: IR ν_{max} 3335, 2851, 1684, 1613, 1462, 145 cm^{-1}; UV λ_{max} nm (log ε) 3.47 (3.95), 272 (4.14), 228 (4.71); ^{1}H and ^{13}C NMR spectra: see Table 1; ESIMS (+) *m/z*: 239.0693 [calcd. for $C_{13}H_{12}NaO_3$ 239.0684, M + Na]$^+$ 217.0855 [calcd. for $C_{13}H_{13}O_3$ 217.0865, MH]$^+$.

4.1.3. Agropyrenone (3)

Agropyrenone (**3**), obtained as amorphous solid had: $[\alpha]^{25}_{D}$ +16.0 (*c* 0.7, MeOH); IR ν_{max} 3374, 1635, 1553, 1460 cm^{-1}; UV λ_{max} nm (log ε) 310 (4.00), 271 (4.23), 216 (4.85); ^{1}H and ^{13}C NMR spectra: see Table 1; HR ESIMS (+) *m/z*: 231.0645 [calcd. for $C_{11}H_{12}NaO_4$ 231.0633, M + Na]$^+$ 209.0803 [calcd. for $C_{11}H_{13}O_4$ 209.0814, MH]$^+$.

4.2. Preparation of agropyrenol derivatives

4.2.1. 3',4'-*O,O'*-Diacetylagropyrenol (4)

Agropyrenol (**1**, 10 mg) was acetylated with pyridine (50 μL) and Ac_2O (50 μL) at room temperature overnight. The reaction was stopped by addition of MeOH and the azeotrope formed by addition of C_6H_6 was evaporated by a N_2 stream. The oily residue (12 mg) was purified by preparative TLC (silica gel, eluent $CHCl_3$-*i*-PrOH (9:1)) to give the 3',4'-*O,O'*-diacetyl derivative (**4**) of agropyrenol as a homogeneous compounds (13.0 mg, R_f 0.91).

Derivative **4** had: $[\alpha]^{25}D$: +7 (0.5); IR ν_{max} 2847, 1733, 1640, 1612, 1571, 1499, 1219 cm^{-1}; UV λ_{max} nm (log ε): 347 (2.59); 272 (2.93), 227 (3.40) . ^1H NMR see Table 2; HR ESIMS (+) *m/z*: 345 $[M+K]^+$, 329 $[M+Na]^+$, 289 $[M+Na\text{-}AcOH]^+$, 246 $[M+Na\text{-}AcOH\text{-}MeCO]^+$.

4.2.2. 3',4'-*O*,*O*'-Isopropylideneagropyrenol (5)

Agropyrenol (**1,** 10 mg) was dissolved in dry Me_2CO (10 mL) and kept in stirred condition with dry $CuSO_4$ (400 mg) under reflux for 2 h. The mixture was then filtered and the solvent evaporated under reduced pressure to give an oily residue which was purified by preparative TLC (silica gel, eluent $CHCl_3$), yielding **5** as a homogeneous compound (11 mg, R_f 0.66). Derivative **5** had: IR ν_{max} 3604, 2868, 1643, 1610, 1572, 1451, 1237 cm^{-1}; UV λ_{max} nm (log ε): 347 (3.30); 274 (3.60), 230 (3.97). ^1H NMR see Table 2; HR ESIMS (+) *m/z*: 285 $[M+Na]^+$, ESIMS (-) *m/z*: 261 $[M-H]^-$.

4.2.3. 4',*O*-Didehydroagropyrenol (6)

Agropyrenol (**1,** 10 mg) dissolved in dry CH_2Cl_2 (3 mL) was oxidized with MnO_2 (82 mg), under stirring at room temperature for 1.5 h. The mixture was then filtered and the solution evaporated under reduced pressure to give an oily residue which was purified by preparative TLC (silica gel, eluent $CHCl_3$-*i*-PrOH (97:3)) giving **6** as a homogeneous compound (3 mg, R_f 0.69). Derivative **6** had: IR ν_{max} 3434, 2851, 1736, 1684, 1646, 1580, 1465 cm^{-1}; UV λ_{max} nm (log ε): 355 (3.05); 285 (3.40), 246 (3.62). ^1H NMR see Table 2; ESIMS (+) *m/z*: 177 $[M+CH_2CO+H]^+$, HR ESIMS (-) *m/z*: 175 $[M\text{-}CH_2CO\text{-}H]^-$.

4.2.4. 7,*O*-Dihydroagropyrenol (7)

Agropyrenol (**1**, 5 mg) was dissolved in MeOH (200 μL) and reduced at room temperature with $NaBH_4$ (7 mg) for 2 h. The mixture was then diluted with water (10 mL), neutralized to

pH 7 with HCl 0.1 N, extracted with EtOAc (4x15 ml), dehydrated with Na_2SO_4 and finally evaporated under reduced pressure giving an oily residue. It was purified by preparative TLC (silica gel, eluent $CHCl_3$-*i*-PrOH (1:1)) yielding **7** as a homogeneous compound (5 mg, R_f 0.74). Derivative **7** had: IR ν_{max} 3395, 1659, 1577, 1462, cm^{-1}; UV λ_{max} nm (log ε): 253 (2.84); 216 (sh). ^{1}H NMR see Table 2; HR ESIMS (+) *m/z*: 263 $[M+K]^+$, 247 $[M+Na]^+$.

4.2.5. 7,7,1'2'-Tetrahydro-7-deoxyagropyrenol (8)

Agropyrenol (**1,** 5 mg) was first dissolved in MeOH (500 μL), then added to a presaturated 10% Pd/C (3 mg) suspension in the same solvent (500 μg), and hydrogenated at room temperature and atmospheric pressure under stirred conditions. After 1 h the reaction was stopped by filtration and suspension was evaporated under reduced pressure. The residue was purified by preparative TLC (silica gel, eluent $CHCl_3$-*i*-PrOH (9:1)) giving **8** as a homogeneous compound (2.5 mg, R_f 0.53). Derivative **8** had: IR ν_{max} 3373, 1585, 1541, 1465 cm^{-1}; UV λ_{max} nm (log ε): 280 (1.81). ^{1}H NMR see Table 2; HR ESIMS (+) *m/z*: 249 $[M+K]^+$, 233 $[M+Na]^+$, ESIMS (-) *m/z*: 209 $[M-H]^-$.

4.2.6. 6,3',4'-*O,O',O"*-Triacetyl derivative (9)

Derivative **8** (8 mg) was acetylated with pyridine (50 μL) and Ac_2O (50 μL) at room temperature for 1.5 h. The reaction was stopped by addition of MeOH. The azeotrope obtained by addition of C_6H_6 was evaporated by a N_2 stream. The oily residue (12 mg) was purified by preparative TLC (silica gel, eluent $CHCl_3$/*i*-PrOH (95:5, v/v)) to give the 7,3',4'-*O,O'*,O"-tiacetyl derivative (**9**) of agropyrenol as a homogeneous compounds (11.0 mg, R_f 0.76). Derivative **9** had: IR ν_{max} 1761, 1734, 1580, 1207 cm^{-1}; UV λ_{max} nm (log ε): 259 (2.46). ^{1}H NMR see Table 2; HR ESIMS (+) *m/z*: 359 $[M+Na]^+$.

4.2.7. 3'-*O*-(*S*)-α-Methoxy-α-trifluoromethyl-α-phenylacetate (MTPA) ester of agropyrenol (10)

(*R*)-(-)-MPTA-Cl (50 μL) was added to agropyrenol (**1**, 5.3 mg) dissolved in dry pyridine (60 μL). The mixture was cooled on ice-bath. After 1 min, the reaction stopped by adding MeOH and the pyridine was removed by a N_2 stream. The residue (5 mg) was purified by preparative TLC ($CHCl_3$-*i*-PrOH (95:5)), yielding **10** as a homogeneous oil (1.1 mg, R_f 0.67). **10** had: IR ν_{max} 3362, 2862, 1706,1646, 1607, 1574, 1495, 1451, 1259 cm^{-1}; UV λ_{max} nm (log ε) 347 (3.95), 272 (4.34), 228 (4.71); ^1H spectrum see Table 3; ESIMS (+) *m/z* 461 $[M+Na]^+$; ESIMS (-) *m/z* 437 $[M-H]^-$.

4.2.8. 3'-*O*-(*R*)-α-Methoxy-α-trifluoromethyl-α-phenylacetate (MTPA) ester of agropyrenol (11)

(*S*)-(+)-MPTA-Cl (50 μL) was added to agropyrenol (**1**, 4.9 mg) dissolved in dry pyridine (60 μL). The reaction was carried out under the same conditions used for preparing **10** from **1**. The purification of the crude residue (4.8 mg) by preparative TLC ($CHCl_3$-*i*-PrOH (95:5)) gave **11** as a homogeneous oil (1.3 mg, R_f 0.64). **10** had: IR ν_{max} 3467, 2851, 1745, 1644, 1608, 1569, 1488, 1448, 1264 cm^{-1}; UV λ_{max} nm (log ε) 350 (3.93), 274 (4.22), 228 (4.64); ^1H spectrum see Table 3; ESIMS (+) *m/z* 461 $[M+Na]^+$; ESIMS (-) *m/z* 437 $[M-H]^-$.

4.2.9. 4'-*O*-(*S*)- α-Methoxy-α-trifluoromethyl-α-phenylacetate (MTPA) ester of agropyrenol (12)

(*R*)-(-)-MPTA-Cl (50 μL) was added to agropyrenol (**1**, 5.3 mg) dissolved in dry pyridine (60 μL). The reaction was carried out under the same conditions used for preparing **10** from **1**. The purification of the crude residue (5.0 mg) by preparative TLC (solvent system A) gave **12** as homogeneous oil (2.3 mg, R_f 0.70). **12** had: IR ν_{max} 3362, 2862, 1706, 1646, 1607, 1574,

1495, 1451, 1259 cm^{-1}; UV λ_{max} nm (log ε) 347 (3.95),272 (4.34), 228 (4.71), ^1H spectrum see Table 3; ESIMS (+) *m/z* 461 $[M+Na]^+$; ESIMS (-) *m/z* 437 $[M-H]^-$.

4.2.10. 4'-*O*-(*R*)- α-Methoxy-α-trifluoromethyl-α-phenylacetate (MTPA) ester of agropyrenol (13)

(*S*)-(+)-MPTA-Cl (50 μL) was added to agropyrenol (4.9 mg) dissolved in dry pyridine (60 μL). The reaction was carried out under the same conditions used for preparing **10** from **1**. The purification of the crude residue (4.8 mg) by preparative TLC ($CHCl_3$-*i*-PrOH (95:5)) gave **13** as homogeneous oil (2.4 mg, R_f 0.67). **13** had: IR ν_{max} 3467, 2851, 1745, 1644, 1608, 1569, 1488, 1448, 1264 cm^{-1}; UV λ_{max} nm (log ε) 350 (3.93), 274 (4.22), 228 (4.64; ^1H spectrum see Table 3; ESIMS (+) *m/z* 461 $[M+Na]^+$; ESIMS (-) *m/z* 437 $[M-H]^-$.

4.3. Production, extraction and purification of phomentrioloxin from *Diaporthe gulyae* culture filtrates

The fungus *Diaporthe gulyae* was grown in 1 L Erlenmeyer flasks containing 300 mL of a defined mineral (Pinkerton and Strobel, 1976). Each flask was seeded with 5 mL of a mycelia suspension and then incubated at 25° C for 4 weeks in the dark. After mycelial removal by filtration, the culture filtrates (4 L) were lyophilized. The residue was then dissolved in 400 mL of distilled H_2O, acidified to pH 2.5 with HCOOH, and extracted with EtOAc (4 x 400 L). The organic extracts were combined, dried by Na_2SO_4 and evaporated under reduced pressure, giving a brownish-red oil residue (937 mg) showing high phytotoxic activity. It was fractionated by silica gel column eluted with $CHCl_3$-*i*-PrOH (9:1), obtaining 11 groups of homogeneous fractions. Fraction groups 5, 6, 9, and 10 proved to be phytotoxic and were further purified. The residues of fraction 5 and 6 were combined (170 mg) and purified by preparative TLC, eluted with $CHCl_3$-*i*-PrOH (9:1), producing four bands. The

third band (81 mg) was scraped off giving a residue which was purified by TLC on reverse phase with H_2O-EtOH (4:6) to afford phomentrioloxin (**14**, 40.3 mg, 10.0 mg/L, R_f 0.32) as a homogeneous solid. It was crystallized by slow evaporation from a MeOH/H_2O (3:1) solution.

4.3.1. Phomentrioloxin (14)

Phomentrioloxin (**14**, Fig. 19), obtained as crystal had: $[\alpha]^{25}_D$: -23 (*c* = 0.4, $CHCl_3$); IR ν_{max} 3385, 2186, 1666, 1628, 1604 cm^{-1}; UV λ_{max} nm (log ε) 259 (4.25), 240 (4.25), 230 (4.25); ^1H and ^{13}C NMR spectra: see Table 4; HR ESIMS (+) *m/z*: 607 $[2M + Na]^+$, 331 $[M + K]^+$, 315.1561 [calcd. for $C_{17}H_{24}NaO_4$ 315.1572, $M + Na]^+$.

4.4. X-ray diffraction study of phomentrioloxin

Colourless block shaped single crystals of phomentrioloxin (**14**) were obtained at ambient temperature by slow evaporation of a MeOH/H_2O (3:1) solution. Cell parameters were obtained from a least-squares fit of the θ angles of 44 reflections in the range $3.818° \leq \theta \leq 18.589°$. A semiempirical absorption correction (multi-scan, SADABS) was applied. The structure was solved by direct methods and anisotropically refined by the full matrix least-squares method on F^2 against all independent measured reflections (SIR97 package) (Altomare et al. 1999) and refined by the full matrix least-squares method on F^2 against all independent measured reflections (SHELXL program of SHELX97 package) (Sheldrick, 2007). The position of hydroxy H atoms was determined from a difference Fourier map and refined according to a riding model. In the absence of significant anomalous scatters, the absolute configuration cannot be determined, Friedel pairs were therefore merged before the final refinement. The final refinement converged to R1 = 0.0546 for 1627 observed reflections having $I > 2\sigma(I)$. Minimum and maximum residual electronic density was -0.211 and 0.236 e/$Å^3$. Crystal data: formula $C_{17}H_{24}O_4$, formula weight 292.36 g/mol, orthorhombic

P $2_12_12_1$, a=4.632(3), b=11.982(7), c=29.48(2)Å, α=β=γ=90°, 6405 collected reflections, 1627 unique reflections.

4.5 Preparation of phomentrioloxin derivatives

4.5.1. 1-*O*-Acetyl-, 1,2-*O,O'*-diacetyl and 1,2,4-*O,O',O''*-triacetyl -phomentrioloxin (15, 16 and 17)

Phomentrioloxin (**14**, 12 mg) was acetylated with pyridine (80 μL) and Ac_2O (80 μL) at room temperature for 5 minutes. 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 (13.7 mg) was purified by preparative TLC eluted with $CHCl_3$-*i*-PrOH (97:3), to give the 1-*O*-acetyl, the 1,2-*O,O'*-diacetyl and the 1,2,4-*O,O',O''*-triacetyl derivatives of phomentrioloxin (**15**, **16** and **17**) as homogeneous compounds (4.1 mg, R_f 0.20; 3.9 mg, R_f 0.40; 3.2 mg, R_f 0.63, respectively). Derivative **15** had: IR ν_{max} 3382, 1714, 1661,1646, 1615, 1269 cm^{-1}; UV λ_{max} nm (log ε): 274 (sh), 257 (3.13). ^1H spectrum: see Table 5; ESIMS (+) *m/z*: 373 $[M+K]^+$. 357 $[M+Na]^+$. Derivative **16** had IR ν_{max} 3397, 1721, 1661,1646, 1615, 1269 cm^{-1}; UV λ_{max} nm (log ε): 273 (sh), 261 (3.24). ^1H spectrum: see Table 5; ESIMS (+) *m/z*: 415 $[M+K]^+$, 399 $[M+Na]^+$. Derivative **17** had: $[\alpha]^{25}_D$: -132 (*c* 0.3, $CHCl_3$); IR ν_{max} 2335, 1747, 1637,1599, 1220 cm^{-1}; UV λ_{max} nm (log ε): 273 (sh), 261 (4.56). ^1H spectrum: see Table 5; ESIMS (+) *m/z*: 441 $[M+Na]^+$.

4.5.2. 1,2-*O,O'*-Isopropylidene of phomentrioloxin (18)

To phomentrioloxin (**14**, 12.0 mg) dissolved in dry acetone (12.0 ml), anhydrous $CuSO_4$ (480.0 mg) was added. The reaction was carried out by reflux under stirring for 2 h, and then stopped by filtration. The solution obtained was evaporated under reduced pressure. The residue (13.4 mg) was purified by preparative TLC, eluted with $CHCl_3$-*i*-PrOH (97:3),

yielding **18** as a homogeneous oil (6.3 mg, R_f 0.53) . **18** had: $[\alpha]^{25}_D$: +21 (*c* = 0.2); IR ν_{max} 3450, 2280, 1725, 1631, 1604 cm^{-1}; UV λ_{max} nm (log ε) 260 (5.01); ^1H NMR: see Table 5; ESIMS (+) *m/z* 687 $[2M+Na]^+$, 355 $[M+Na]^+$

4.5.3. 4',*O*-Didehydrophomentrioloxin (19)

Phomentrioloxin (**14,** 9 mg) dissolved in dry CH_2Cl_2 (3 mL) was oxidized with MnO_2 (65 mg), under stirring at room temperature for 5 h. The mixture was then filtered and the solvent evaporated under reduced pressure to give an oily residue, which was purified by preparative TLC (silica gel, eluent $CHCl_3$/*i*-PrOH (95:5, v/v)) giving **19** as a homogeneous compound (3 mg, R_f 0.56). Derivative **19** had: IR ν_{max} 3385, 2186, 1674, 1607, 1594 cm^{-1}; UV λ_{max} nm (log ε): 283 (3.51); ^1H NMR see Table 5; ESIMS (+) *m/z*: 313 $[M+Na]^+$.

4.5.4. 1',1',2',2',3',8',6',7'-Octahydrophomentrioloxin (20)

Phomentrioloxin (**14,** 8 mg) was first dissolved in MeOH (500 μL), then added to a presaturated 5% Rd/C (3 mg) suspension in the same solvent (500 μL), and hydrogenated at room temperature and atmospheric pressure under stirring conditions. The reaction was stopped after 15 min by filtration, and the solvent evaporated under reduced pressure; the residue (13.5 mg) was purified by preparative TLC (silica gel, eluent petroleum ether/Me_2CO (7:3) giving **20** as a homogeneous compound (3 mg, R_f 0.35). Derivative **20** had: IR ν_{max} 3410, 1666, 1460 cm^{-1}; UV λ_{max} nm (log ε): < 220. ^1H NMR see Table 5; ESIMS (+) *m/z*: 339 $[M+K]^+$, 323 $[M+Na]^+$.

4.5.5. Conversion of phomentrioloxin into 2-methoxy-5-(3-methylene-oct-6-en-1-ynyl)-hex-4-ene-1,3,6-triol (21)

Phomentrioloxin (**14**, 7 mg) dissolved in MeOH (10.2 mL) was oxidized with an aqueous solution (3.2 mL) of $NaIO_4$ (8 mg) under stirring at room temperature in the dark. After 1 h, ethylene glycol (0.6 ml) and cold acetone (3.2 mL) were added and the precipitated $NaIO_3$ was removed by filtration. The solution was extracted with CH_2Cl_2 (4x15mL) and the extract evaporated under reduced pressure. The residue, dissolved in MeOH (2 mL), was reduced with $NaBH_4$ (8 mg) for 1 h at room temperature. The reaction was stopped by neutralization with 1 M HCl (1 mL) and the aqueous solution extracted with CH_2Cl_2. The extract was dehydrated (Na_2SO_4) and purified by TLC (silica gel, eluent $CHCl_3$-*i*-PrOH (85:15) giving **21** as a homogeneous compound (3 mg, R_f 0.57). Derivative **21** had: IR ν_{max} 3390, 1643, 1615, 1573 cm^{-1}; UV λ_{max} nm (log ε): 274 (sh); 256 (3.15), 256 (3.15). ^1H NMR see Table 5; ESIMS (+) *m/z*: 317 $[M+Na]^+$.

4.5.6. 4-*O*-(*S*)-α-Methoxy-α-trifluoromethyl-α-phenylacetate (MTPA) ester of derivative 18 (22). (*R*)-(-)-MPTA-Cl (10 μL) was added to derivative **18** of phomentrioloxin (2.0 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 $CHCl_3$, yielding **22** as a homogeneous oil (R_f 0.69, 2.1 mg,). It had: IR ν_{max} 2389, 1750, 1451, 1381, 1244 1169 cm^{-1}; UV λ_{max} nm (log ε) 260 (4.56); ^1H NMR spectrum see Table 6; ESIMS (+) *m/z* 571 $[M+Na]^+$, 413 $[M+Na-M_2CO]^+$.

4.5.7. 4-*O*-(*R*)-α-Methoxy-α-trifluoromethyl-α-phenylacetate (MTPA) ester of derivative 18 (23). (*S*)-(+)-MPTA-Cl (10 μL) was added to to derivative **18** of phomentrioloxin **(**2.0 mg)

dissolved in dry pyridine (20 μL). The reaction was carried out under the same conditions used for preparing **22**. The purification of the crude residue (2.2 mg) by preparative TLC, eluted with $CHCl_3$, gave **23** as homogeneous oil (R_f 0.69, 1.6 mg). It had: IR ν_{max} 2377, 1750, 1451, 1377, 1237, 1168 cm^{-1}; UV λ_{max} nm (log ε) 261 (4.85); ^1H NMR spectrum see Table 6; ESIMS (+) *m/z* 571 $[M+Na]^+$, 413 $[M+Na-M_2CO]^+$.

4.6. Biological assays

4.6.1 Phytotoxic activity

Culture filtrates, organic extracts, chromatographic fractions and pure compounds were assayed on weedy plants: *Mercurialis annua* L., *Chenopodium album, Setaria viridis* (L.) P. Beauv., *Cirsium arvense, Sonchus arvensis, Carthamus lanatus, Sonchus oleraceus* L. The plants were produced from pieces of underground shoots or seeds and grown in a greenhouse. Crude organic extract, chromatographic fractions and pure compounds were dissolved in a small amount of MeOH and then brought up to desirable concentration with distilled H_2O (1-2 mg/mL). The final concentration of MeOH in test solutions was 2% v/v that is non toxic to leaves of all plants used as control. Droplets (10-20 μl) of the test solution were applied on the discs and then incubated in transparent plastic boxes at 24° C under 12 h photoperiod. The following bioassays were performed:

4.6.1.1. Leaf disk puncture assay

Pure compounds were tested at 2 mg/mL by applying 20 μL of solution to detached leaves previously punctured with a needle. The discs (10 mm diam.) were cut off from well-expanded leaves, placed on moistened filter paper and punctured with a sharp needle in the centre. 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, 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.

4.6.1.2. Seed germination

The possible capability to inhibit seed germination was assayed on seeds of *Setaria viridis*. Briefly, seeds were first sterilized with sodium hypochlorite (1% × 15 min) and then washed several times with sterile distilled water. Around 100 seeds were placed in each Petri dish on paper dishes (Whatman No. 4) imbibed with the metabolite solution (1 mL, 10^{-3} M) and incubated at 25 °C in the dark. Three replications were prepared per each compound. The number of germinated seeds were counted 5 days after treatment and expressed as the germination percentage in comparison to the untreated control.

4.6.1.3. Rootlet elongation

This assay aimed at evaluating the possible inhibition of the rootlet growth, and it was carried out using tomato seeds. Briefly, tomato seeds were pre-treated with sodium hypochlorite (1% × 10 min.) and placed in Petri dishes on filter paper disks imbibed with sterile distilled water for 5 days. Thus, 10 pre-germinated and uniform length seedlings were transferred to smaller dishes each containing the metabolite solution (2 mL, 10^{-3} M). Dishes were transferred to a growth chamber under continuous light. Three replicates were prepared for each sample. After 4 days, the rootlet length was measured and compared to the proper untreated control.

4.6.1.4 Chlorophyll degradation and frond growth

Tests were carry out on the aquatic plant *Lemna minor* L. at concentration between 6.85 mM and 68.5 µM, by adapting a protocol already described (Vesonder et al., 1992), for the possible capability to degrade chlorophyll and inhibit frond growth. Briefly, the wells of sterile, polystyrene 96-well microtitre plates were filled with 50 µL aliquot of solutions

containing the metabolites to be tested, at the concentration reported above. One frond of actively growing axenic *L. minor* was placed into each well. Control wells were included in each plate. Four replications were prepared for each compound. The plates were incubated in a growth chamber with 12/24 h fluorescent lights and observed daily up to 4 days. One day after the application of the test solution, 100 µL of distilled H_2O was added to each well. Appearance of necrosis or chlorosis was assessed visually by comparison of the treated plants with the control appearance. Moreover, plantlet fresh weight was measured, and chlorophyll contents were determined by using the protocol described (Marr et al. 1995).

4.6.2. Antimicrobial bioassay

The antimicrobial activity was tested against three microorganisms using an agar diffusion assay according to the protocol previously described (Bottalico et al. 1990). In particular, the antifungal activity was tested on *Geotrichum candidum* Link grown on potato dextrose agar (PDA), whereas the antibiotic activity was assayed against *Bacillus subtilis* (a gram-positive bacterium) grown on tryptic glucose yeast agar (TGYA, Biolife, Bothell, WA) and *Escherichia coli* (Migula) Castell. & Chalm. (a gram negative bacterium) grown on Luria-Bertani (LB) agar (Sigma, St. Louis, MO). Up to 60 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 one day after the application.

4.6.3. Zootoxic activity assay

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

5. RESULTS AND DISCUSSION

5.1. Purification and chemical characterization of phytotoxins from *Ascochyta agropyrina* culture filtrates

A previous study on *A. agropyrina* var. *nana* led to the isolation of a main phytotoxin from the solid culture of this fungus and its identification as papyracillic acid (Evidente et al., 2009). However, when grown in a liquid medium, the fungus produces bioactive metabolites other than papyracillic acid. For this reason the fungus was grown in liquid cultures and the organic extract, showing a high phytotoxic activity was purified by a combination of CC and TLC (normal and reverse phase) giving three pure metabolite (Scheme 1). Two of them proved to be phytotoxic.

Agropyrenol (**1**; Fig. 16) was isolated from the medium polar fraction. It has a molecular weight of 222 and matches a molecular formula of $C_{12}H_{14}O_4$, as deduced from its HR-ESIMS spectrum (Fig. 17).

The detailed analysis of the ^{1}H NMR (Table 1) and COSY (Berger and Braun, 2004) spectra (Fig. 18 and 19 respectively) reveals the presence of a typical pattern of 1,2,3-trisubstituted benzene ring whose protons appeared as a doublet of doublets (J = 8.8 and 7.6 Hz) and two doublets (J = 7.6 Hz and 8.4 Hz) at the typical shift values of δ 7.44, 6.93 and 6.90 (H-4, H-5 and H-3) (Pretsch et al., 2000). The presence of two singlets at δ 11.9 and 10.3 suggested the presence of an aldehyde hydrogen H-bonded to an *ortho*-positioned phenolic group, with the latter also *ortho*-positioned to H-5 (Pretsch et al., 2000). This was also confirmed by the typical absorption band observed in both IR (Nakanishi and Solomon, 1977) and UV (Scott, 1964) spectra (Fig. 20 and 21 respectively). Furthermore, the proton systems of a 3,4-dihydroxy-penten-1-yl residue, which consequently should be bonded to the C-6 of the benzene ring, were also observed. In particular, the benzyl proton (H-1') of a *trans*-disubstituted double bond, which resonated as a doublet (J = 15.6 Hz) at the typical chemical

shift value of δ 7.23, coupled with the other olefinic proton (H-2'), which appeared as a doublet of doublets (*J* = 15.6 and 6.1 Hz) at δ 6.12, being also coupled with the proton (H-3') of the adjacent secondary hydroxylated carbon. The latter (H-3') appeared as a doublet of doublets (*J* = 6.4 and 6.1 Hz), being also coupled with the proton (H-4') of its adjacent secondary hydroxylated carbon which resonated as a quintet (*J* = 6.4 Hz) at δ 3.71, as this in turn is also coupled with the terminal methyl group, which resonated as a doublet (*J* = 6.4 Hz) at δ 1.27 (Pretsch et al., 2000). The 1,2,3-trisubstituted benzene ring, as well as the aldehydic group and the 3,4-dihydroxy-1-pentenyl found as substructures in agropyrenol were confirmed by the data of its ^{13}C NMR spectrum (Table 1; Fig. 22) and the couplings observed in the HSQC spectrum (Fig. 23) (Berger and Braun, 2004), which allowed to assign the chemical shifts to all the protonated carbons. In particular, the aldehydic carbonyl appeared at the very typical chemical shift value of δ 195.2, as well as the three secondary aromatic carbons observed at δ 137.2, 118.8 and 117.4 (C-4, C-5 and C-3), respectively. The remaining hydroxylated and quaternary benzene carbons (C-6 and C-2) were at 162.8 and δ 142.0, respectively (Breitmaier and Voelter, 1987) as also deduced from the coupling observed in the HMBC spectrum (Table 2; Fig. 24) (Berger and Braun, 2004), which also supported the location of the 3,4-dihydroxy-penten-1-yl side chain at C-6. The olefinic (C-1' and C-2'), the secondary hydroxylated (C-3' and C-4'), and the terminal methyl (C-5') carbons appeared at the typical chemical values of δ 126.9, 135.9, 77.0, 70.8 and 19.3, respectively (Breitmsaier and Voelter 1987). The location of the three substituents on C-1, C-2 and C-6 was confirmed by the significant couplings observed in the HMBC spectrum and in particular those observed between the aldehydic and the hydroxy protons and C-5 and C-6, H-2' with C-1, H-1' and H-2'with C-2. So that the structure in Fig. 16 (**1**) was assigned to agropyrenol, the main toxin produced by *A. agropyrina* var. *nana* in liquid culture.

This structure was confirmed by all the other couplings observed in the HMBC and NOESY (Table 2 and 3) spectra. Indeed, in this latter spectrum the expected coupling between H-4 and both H-5 and H-3 as well as those of some protons of the 3,4-dihydroxypentyl side chain themselves were observed. In agreement with structure (Fig. 16), the HR-ESIMS spectrum (Fig. 17) showed the sodium cluster at *m/z* 245.1601 $[M + Na]^+$.

The absolute configuration was determined by applying the modified Mosher's method (Othani et al., 1991). By reaction with the *R*-(-)-α-methoxy-α-trifluoromethyl-α-phenylacetate (*R*-(-)-MTPA) and *S*-(+)-MTPA chlorides, agropyrenol was converted into the corresponding diastereomeric *S*-MTPA and *R*-MTPA monoesters at C-3' (**10** and **11** respectively; Fig. 25), whose spectroscopic data were consistent with the structure assigned to agropyrenol. The comparison between the ^{1}H NMR data (Table 4) of the *S*-MTPA ester (**10**; Fig. 26) and those of the *R*-MTPA ester (**11**; Fig. 27) [Δδ (**10**-**11**): OH-6 +0.04; HCO-7 +0.101; H-4 +0.004; H-3 +0.018; H-1' +0.04; H-5 +0.038; H-2' +0.108; H-4' -0.018; Me-5' -0.062] allowed to assign an *R*-configuration to C-3'. Applying the same method, agropyrenol was also converted into the diastereomeric *S*-MTPA and *R*-MTPA monoesters at C-4' (**12** and **13** respectively; Fig. 25), whose spectroscopic data were consistent with the structure assigned to agropyrenol. The comparison between the ^{1}H NMR data (Table 4) of the *S*-MTPA ester (**12**; Fig. 28) and those of the *R*-MTPA ester (**13**; Fig. 29) [Δδ (**12**-**13**): OH-6 -0.005; HCO-7 –0.057; H-4 -0.016; H-3 -0.012; H-1' -0.098; H-5; -0.057; H-2' -0.106; H-3' -0.056; Me-5' +0.069] allowed to assign an *R*-configuration also at C-4'. So the agropyrenol could be reformulated as ((3'*R*,4'*R*)-dihydroxy-pent-1-enyl)-6-hydroxy-benzaldehyde.

^{1}H and ^{13}C NMR spectra (Fig. 18 and 22 respectively) and other spectroscopic properties (IR and UV) (Fig. 20 and 21 respectively) of agropyrenol are very similar to those reported for sordarial, a hexaketide isolated together with some other closely related metabolites from *Sardaria macrospora*, but for which no biological activity was reported (Bouillant et al., 1989). In addition, sordarial was also isolated together with three new 2-

pyrones from three Ascomycetes (*Gelasinospora heterospora*, *G. multiforis*, and *G. logispora*) (Fujimoto et al., 1999) and its immunosuppressive activity against concanavalin A (T cell) and lipopolysaccharide-induced proliferations of mouse splenic lymphocytes (B-cells) (Fujimoto et al., 2006) was reported. The same authors, applying the Mosher ester method also determined the absolute configuration of sordarial as 3'*R* and 4'*S* (Fujimoto et al., 1999).

However, after a more detailed comparison of the ^{1}H NMR of **1** to that of sordarial recorded using $CDCl_3$ as solvent (Fujimoto et al., 1999), a significant difference in the chemical shifts of the protons of the two methyne hydroxylated carbon of the side chain H-3' and H-4' was observed. These appeared significantly upfield shifted (Δδ 0.24 and 0.30). This observation suggested that **1** could be a diastereomer of sordarial. This hypothesis was confirmed by measuring the optical rotation of **1** under the same conditions as those reported in the literature. Indeed, when the optical rotation of **1** (levorotatory, $[\alpha]^{25}_{D}$ -47) was compared with that of sordarial (dextrorotatory, $[\alpha]^{20}_{D}$ +18.2 (Bouillant et al., 1989); $[\alpha]^{20}_{D}$ +22 (Fujimoto et al. 1999)) it appears substantially different in value and opposite in sign. The *R,R*-configuration assigned to C-3' and C-4' is different from that previously assigned to sordarial (3'*R*,4'*S*) and thus their diastereomeric relationship was confirmed.

To determine the relationships between the structure of agropyrenol and its biological properties, identify the active sites of the compound, and possibly, increase its toxicity, six derivatives of the main fungal toxin were prepared. Agropyrenol was converted into the corresponding 3′,4′-*O*,*O*′-diacetyl derivative (**4**; Fig. 30) by routine acetylation carried out with acetic anhydride and pyridine, which showed the reversible modification of the diol system of the 3,4-dihydroxy-1-pentenyl residue. This was confirmed by the ^{1}H NMR spectrum (Fig. 31, Table 5). In fact, this latter showed two singlets of MeCO at δ 2.14 and at δ 2.07, the downfield shifts (Δδ 1.88, and 1.78) and the different multiplicity of H-3' and H-4' resonating as a double doublet (J = 6.8, 6.5 and 4.1 Hz) and quintet (J = 6.5 Hz), at δ 5.98 and

5.49, respectively. The HRESI-MS spectrum of **4** (Fig. 32) showed the potassium and sodium clusters and the pseudomolecular ions at *m*/*z* 345, 329, 289 and 246 (see experimental). Its conversion into the parent compound at physiological pH frequently occurs in other natural metabolites, and it is known as "lethal metabolism" (Hassal, 1990). A different modification of the same diol system was obtained by conversion of agropyrenol into the corresponding 3′,4′-*O*,*O*′-isopropylidene derivative (**5**; Fig. 30). It was obtained by reaction of compound **1** with dry Me_2CO and dry $CuSO_4$. The ^{1}H NMR data of derivative **5** (Fig. 33, Table 5) differed from those of **1** for the presence of two singlets of the isopropylidene group at δ 1.48 and 1.45, respectively. Furthermore, the spectrum indicate downfield shifts (Δδ 0.04 and 0.19) and the different multiplicity of the double doublet (*J* = 8.4, 6.6 and 1.0 Hz) and the double quartet (*J* = 8.4 and 6.0 Hz) of H-3' and H-4' engaged in the isopropylidene ring. The HR-ESIMS spectrum of **5** (Fig. 34) showed the sodium cluster and the molecular ion at *m*/*z* 285 and 261 (see experimental). Further, different modifications of the 3,4-dihydroxy-1-pentenyl residue were obtained by oxidation of agropyrenol with MnO_2. Although this reaction yielded three oxidized derivatives, only the major one (**6**, Fig. 30), showing the oxidation of the secondary hydroxyl group at C-4′, was purified. The ^{1}H-NMR spectrum (Fig. 35, Table 5) of **6**, compared to **1**, showed the lack of the proton (H-4') of the secondary hydroxylated carbon (C-4') while the proton H-5' resonated as singlet at δ 1.25 and the proton H-3' resonated as multiplet at δ 4.21. The HR-ESIMS spectrum of **6** (Fig. 36) showed the molecular ions at *m*/*z* 177 and 175 (see experimental). The other two oxidized derivatives were obtained as minor products of this reaction. The ESI−MS of their mixture proved that they are mono-oxidized at C-3′ and dioxidized at C-3′ and C-4′, respectively. Unfortunately, they were not purified, despite the attempts made using high-performance liquid chromatography (HPLC). Furthermore, opposite to what is currently reported (Allinger et al., 1976) the mono-oxidized derivative **6** was also obtained when compound **1** underwent a reaction with $NaIO_4$, with the aim to obtain an oxidative cleavage of the diol system. Anyway, further mechanistic studies

would be needed to clarify the course of this latter reaction. As expected, the reduction by $NaBH_4$ converted compound **1** into the corresponding 7,O-dihydro derivative (**7**; Fig. 30), with the conversion of the aldehyde group into the corresponding primary hydroxy group. The ^{1}H-NMR spectrum (Fig. 37, Table 5) of **7**, compared to **1**, showed the lack of the proton (H-7) of the aldehyde group, but also the significant lack of the characteristic band of hydroxyl group on C-5, no more involved in the hydrogen bond. The proton H-7 resonated as singlet at δ 5.00. The HR-ESIMS spectrum of **7** (Fig. 38) showed the potassium and sodium clusters at *m*/*z* 263 and 247 (see experimental). The catalytic hydrogenation carried out in MeOH with 10% Pd/C at atmospheric pressure and room temperature allowed us to obtain the 7,7,1′,2′-tetrahydro-7-deoxy derivative (**8**; Fig. 17), which has not only the saturation of the double bond of the 3,4-dihydroxy-1-pentenyl residue, as expected, but also the complete and unusual reduction of the aldehyde group to the corresponding methyl group. The ^{1}H-NMR spectrum (Fig. 39, Table 5) of **8**, compared to **1**, showed the lack of the proton (H-7) of the aldehyde group, but also the significant lack of the characteristic band of hydroxyl group on C-5, no more involved in the hydrogen bond, while the proton H-7 resonated as singlet at δ 2.21. Its ^{1}H NMR spectrum (Fig. 39, Table 5) also showed the presence of two double double doublets at δ 2.86 and 2.70 due to H_2 C-1' and two multiplets at δ 1.75 and 1.68 due to H_2 C-2'. The HR-ESIMS spectrum of **8** (Fig. 40) showed the potassium and sodium clusters and the pseudomolecular ion at *m*/*z* 249, 233 and 209 (see experimental). This latter compound was converted into the 6,3′,4′-O,O′,O″-triacetyl derivative (**9**; Fig. 17) by routine acetylation with pyridine and acetic anhydride, already used to convert compound **1** into derivative **4**. Its ^{1}H NMR spectrum (Fig. 41, Table 5) differed from that of **1** for the downfield shifts (Δδ 0.24, 1.67, and 1.42) of H-5, H-3' and H-4' resonating at δ 7.02, 5.06, and 5.06, respectively. Finally, the presence of the three singlets at δ 2.11, 2.09, and 2.07 respectively, due to the

three acetyl groups were also observed. Its HR-ESIMS spectrum (Fig. 42) showed the sodium cluster at *m/z* 359.

Two other metabolites were isolated from the relatively less polar fractions of the initial column and, were named agropyrenale and agropyrenone, respectively (**2** and **3**, Fig. 16).

Agropyrenale (**2**; Fig. 16) proved to have a molecular weight of 216 associated with molecular formula of $C_{13}H_{12}O_3$ as deduced from its HR-ESIMS (Fig. 43), with 8 unsaturations and therefore consistent with the presence of a bicyclic naphthalene carbon skeleton bearing an aldehyde, and a methoxy group hydrogen bonded with an *ortho*-positioned phenolic hydroxy group. These conclusions were made after the investigation of the ^{1}H and ^{13}C NMR spectra (Table 1; Fig. 44 and 45 respectively), which showed typical singlets at δ 12.4, 10.2/189.3 and 3.84/63.6 for the HO-C(7), HCO-9 and MeO-C(8), respectively (Pretsch et al., 2000; Breitmaier & Voelter 1987). The presence of these functionalities was also confirmed by the typical absorption bands observed in the IR and UV spectra (Fig. 46 and 47 respectively) (Nakanishi and Solomon, 1977; Scott, 1964). The ^{1}H NMR and COSY spectra also showed two pairs of two doublets (J = 8.4 Hz) and (J = 9.8 Hz) at the δ 7.03 and 6.52 (H-5 and H-6) and 6.13 and 5.28 (H-2 and H-3). These values are typical chemical shifts of *ortho*-located aromatic protons belonging to a substituted naphthalene system, and a methyl group resonating as a singlet at δ 2.34 (Pretsch et al. 2000). The coupling observed in the HSQC allowed for the assignment of the chemical shifts to their corresponding carbons in the ^{13}C NMR spectrum at δ 134.1, 126.4, 121.8, 109.6, and 15.5 (C-5, C-3, C-2, C-6, and Me-10, respectively). Furthermore, the couplings observed in HMBC (Table 2) led to the assignment of the signals at δ 164.0, 156.0, 155.0, 149.0, 129.5 and 112.0, to the quaternary carbons C-7, C-8, C-1, C-4, C-4a and C-8a, respectively. The couplings observed in the same spectrum also allowed to locate the hydroxy, aldehyde, methoxy and

methyl groups at C-7, C-1, C-8 and C-4, respectively and to assign to agropyrenale the structure of 7-hydroxy-8-methoxy-4-methyl-naphthalene-1-carbaldehyde (**2**).

This structure was also confirmed from the data of its NOESY and HR-ESIMS spectra. The NOESY spectrum (Table 3) showed the expected couplings between the two pairs of aromatic protons but also the significant ones between H-5 and Me-10, and H-9 and MeO, respectively. The HR-ESIMS (Fig. 43) showed the sodium cluster and the pseudomolecular ion at *m/z* 239.0693 $[M + Na]^+$ and 217.0855 $[MH]^+$.

Bioactive natural products containing the naphthalene skeleton are well-known as plant and fungal metabolites (Turner and Aldrige, 1981; Dewick, 2009; Osbourn and Lanzotti, 2009) and some of them are also well known as fungal phytotoxins (Andolfi et al., 2011).

Agropyrenone (**3**; Fig. 16) has a molecular weight of 208 and a molecular formula of $C_{11}H_{12}O_4$ as deduced from its HR-ESIMS (Fig. 48), with 6 unsaturations, and therefore consistent with the presence of a benzofuranone carbon skeleton bearing two hydroxy and two methyl groups. This was concluded based on the investigation of the ^1H and ^{13}C NMR spectra (Table 1; Fig. 49 and 50 respectively), which showed typical singlets at δ 2.14/10.7 and 2.12/8.2 (M-9 and Me-10, respectively), while the γ-lactone carbonyl (C-2) appeared at δ 171.8 (Pretsch et al., 2000; Breitmaier and Voelter 1987). The presence of these functionalities was also confirmed by the typical absorption bands observed in the IR and UV spectra (Fig. 51 and 52 respectively) (Nakanishi and Solomon, 1977; Scott , 1964). The ^1H NMR and COSY spectra also showed a doublet (J = 7.1 Hz) of a third methyl group (Me-8) resonating at δ 1.18 which coupled with the proton of the methine carbon (C-3) of the furanone ring, appearing as quartet (J = 7.1 Hz) at δ 3.23. The couplings observed in the HSQC spectrum allowed for the assignment of the chemical shifts to their corresponding carbons (Me-8 and C-3) at δ 17.5 and 40.0, respectively, as well as the coupling observed in

the HMBC spectrum (Table 2) to those of the quaternary carbons of the benzene ring C-5 and C-7, which gave an overlapped signal, and C-7a , C-3a and C-6 at δ 162.1, ans 161.3, 142.3, and 110.1, respectively. The same couplings also led to the location of the two hydroxy and methyl groups at C-5 and C-7, and C-4 and C-6, respectively.

These results allowed to assign to agropyrenone the structure of 5,7-dihydroxy-3,4,6-trimethyl-3*H*-benzofuran-2-one (**3**). This structure was confirmed by the data of the NOESY and HR-ESIMS spectra. Beside the expected coupling between Me-8 and H-3, the NOESY spectrum (Table 3) showed also the significant one from this latter and Me-9. The HR-ESIMS (Fig. 48) showed the sodium cluster and the pseudomolecular ion at *m/z* 231.0645 $[M + Na]^+$ and 209.0803 $[MH]^+$, respectively.

Benzofuranones are quite common as naturally occurring compounds, and cyclopaldic acid, a pentasubstituted iso-benzofuranone phytotoxin produced by *Seiridium cupressi* (Graniti et al, 1992), can be used as an example.

5.2. Biological activity of phytotoxins from *Ascochyta agropyrina* culture filtrates

5.2.1. Biological activity of agropyrenol and its derivatives

The phytotoxic activity of agropyrenol and its derivatives (**1**, **4**-**9**) was investigated through leaf puncture, seed germination, rootlet elongation, chlorophyll degradation and frond growth assays on non-host plants.

When assayed on leaves of different weed species (*C. album, C. arvense, M. annua, S. viridis* and *S. oleraceus*) at 2 mg toxin/mL of solution, agropyrenol proved to be highly phytotoxic, causing the fast appearance of large necrotic spots (7-8 mm diameter).

Assayed by leaf puncture on detached leaves, derivatives **4** and **5** caused severe to medium necrosis to weedy dicot plants. Derivatives **6**–**9** caused only moderate to nil effects on the same plants, whereas none of the compounds tested had effects on *S. viridis*.

From the analyses of results, it is possible to observe that all of the non-reversible modifications of the diol system of the 3′,4′-dihydroxypentenyl side chain at C-2 present in derivative **6** as well as that of the aldehyde group at C-1 present in derivatives **8** and **9** induced strong reduction or total loss of phytotoxicity. It is interesting to note that derivative **7**, in which only the aldehyde group at C-1 was reduced, produced some phytotoxicity on three of the five plants tested, while the activity was completely lost when also the 1,2-double bond of the 3,4-dihydroxypentenyl side chain was reduced, as in derivatives **8** and **9**. The activity showed by the diacetyl derivate of agropyrenol was not surprising, as cited above; it was probably hydrolyzed into compound **1** at physiological pH according to the "lethal metabolism" (Hassal, 1990). A similar mechanism of hydrolysis could also convert derivative **5** into compound **1** and, therefore, justify its phytotoxicity. In fact, acetonide is stable at basic pH but hydrolyzes in acid conditions (Allinger et al., 1976).

The ability of agropyrenol and its derivatives to inhibit germination and root growth in seeds of *Setaria viridis* and *Lycopersicon esculentum* was investigated. When tested on seeds of *S. viridis*, particularly derivatives **5** and **4** caused a strong reduction of germination (60 and 48% reduction compared to the control, respectively). Agropyrenol (**1**) and derivative **8** had a modest effect (around 20−30%), whereas the other derivatives had a very modest or nil effect (Table 6).

Almost the same effects were observable in both the root elongation assay and the chlorophyll assay, in which agropyrenol showed a medium activity meanwhile derivatives **5** and **4** again proved to be the most effective compounds, causing a severe depletion of rootlet growth on germinated seeds of *L. esculentum* (90 and 69% reduction compared to the control, respectively, as deducible from Table 6) and an effective reduction in the chlorophyll content of *L. minor* fronds (67 and 31% reduction in comparison to the control, respectively; Table 6). All the compounds were tested at 10^{-3} M. These results suggest that derivatives **4** and **5**, which are less polar than compound **1**, could be absorbed and pass across the cell membranes

of the tested plants, before converting into compound **1** as described above. Derivative **5** was active also in most of the bioassays on organisms other than plants. Indeed, it caused 88% mortality of brine shrimp larvae in the zootoxicity bioassay and a slight inhibition of fungal and Gram-positive bacterium growth. In these latter assays, only derivative **8** caused a negligible mortality to *A. salina* larvae, whereas agropyrenol and all of the other derivatives were inactive (Table 6).

Probably to justify the zootoxic and antimicrobial activities of derivative **5** compared to compound **1**, it could be possible to invoke a similar mechanism to that proposed for its behavior in germination, root elongation, and chlorophyll content bioassays.

5.2.2. Biological activity of agropyrenale and agropyrenone

When assayed on leaves of different weed species (i.e., *Chenopodium album*, *Mercurialis annua* and *Setaria viridis*) at 2 mg toxin/mL of solution, agropyrenale showed a medium activity, with the appearance of small necrosis (1-2 mm), whereas agropyrenone was not active at all (Table 7). Although the number of plant species tested was quite limited, the phytotoxicity seems to be not specific and broad, as both dicot and monocot species were affected. None of the compounds caused any growth inhibition of both Gram-(+) (*Bacillus subtilis*) and Gram-(-) (*Escherichia coli*) bacteria when the new compounds were preliminarily tested for antibiotic activity up to 100 µg/diskette (Table 7). Furthermore, these compounds had neither fungitoxic or fungistatic activities when applied up to 100 µg/diskette to *Geotrichum candidum* (Table 7). Finally, no zootoxicity was found against *Artemia salina* larvae when the compounds were tested at 50 µg/well (Table 7).

5.3. Purification and chemical characterization of phytotoxins from *Diaporthe gulyae* culture filtrates

The liquid culture of *D. gulyae* was exhaustively extracted as reported in the experimental section (Paragraph 4.3). The organic extract, showing a high phytotoxic activity, was purified by a combination of CC and TLC guided by biological assays (Scheme 2). The main phytotoxic metabolite was obtained as a homogeneous solid. It crystallized as white needles from a solution of MeOH/H_2O (3:1). The preliminary ^{1}H and ^{13}C NMR (Fig. 53 and 54 respectively) investigations proved that it contains double bonds and hydroxy groups and to be a novel metabolite, to which the name phomentrioloxin was assigned (**14**, Fig. 55).

Phomentrioloxin has a molecular weight 292, due to its molecular formula $C_{17}H_{24}O_4$ deduced by its HR-ESIMS (Fig. 56), consistent with six hydrogen deficiencies. These latter were also confirmed by the typical bands observed in the IR spectrum (Fig. 57) for the olefinic groups and hydroxy groups. The same spectrum also showed a typical band for the presence of at least one alkyne group (Nakanishi and Solomon, 1977). The UV spectrum (Fig. 58) showed absorptions maxima of an extended conjugated chromophore (Scott, 1964).

The detailed investigation of the ^{1}H NMR spectrum (Table 8; Fig. 53) showed the presence of an olefinic proton (H-5) appearing (d, J = 4.0 Hz) at the typical chemical value of δ 6.16. In the COSY spectrum (Fig. 59) (Berger & Braun, 2004) this latter coupled with a proton (H-4) of the secondary hydroxylated carbon (C-4) resonating as doublets of doublet doublets (J = 6.1, 4,1 and 4.0 Hz) at δ 4.51, being also coupled with the protons of the geminal hydroxy group a doublet (J = 6.1 Hz) at δ 2.64 and the proton (H-3) of another adjacent secondary oxygenated carbon (C-3). H-3, resonating as a double doublet (J = 7.9 and 4.1 Hz) at δ 3.70, in turn coupled with the proton (H-2) of another adjacent hydroxylated secondary carbon (C-2) appearing as doublets of double doublets (J = 7.9, 3.6 and 2.0 Hz) at δ 4.21. This latter also coupled with the proton of the geminal hydroxy group (d, J = 2 Hz) at δ 2.68 and with the proton (H-1) of the third adjacent secondary hydroxylated carbon (C-1).

H-1 appeared as a broad singlet at δ 4.35. The same ^{1}H NMR spectrum also showed a further doublet (J = 1.4 Hz) of a hydroxy group and the singlet of a methoxy group linked to C-1 and C-3 at δ 2.65 and 3.55, respectively.

These results agreed with the presence in **14** of a 1,2,4-trihydroxy-3-methoxycyclohexene ring. Consequently, the polyunsaturated side chain constituted by the remaining 10 carbons should be attached to C-6 of this pentasubstituted cyclohexene ring. This suggested a terpenoidal origin of this second partial moiety. Indeed, the ^{1}H NMR and COSY spectra (Fig. 53 and 59 respectively) showed the presence of: 1) two coupled doublets (J = 1.5 Hz) at δ 5.40 and 5.31, respectively, and representing typical chemical shift values for protons of an olefinic methylene group (H_2C-8'); 2) the multiplets of two coupled methylene groups (H_2C-4'and H_2C-5') at δ 2.22 and 1.64, respectively; 3) and singlets of two vinylic methyl groups (Me-9' and Me-10') at δ 2.22 and 1.85, respectively. The remaining two carbons of the geranyl residue attached to C-6 of the cyclohexene ring as well as this latter carbon are quaternary carbons resonating in the ^{13}C NMR spectrum (Table 8; Fig. 54) at δ 135.4, 87.3, and 92.4 (C-6, C-1' and C-2'), typical chemical shift values of olefinic and alkyne carbons (Breitman & Voelter, 1987). The geranyl nature of the side chain attached was delineated by several long range couplings observed between the carbons and the protons of this moiety, as observed in the HMBC spectrum (Table 8; Fig. 60). The presence of these two joined moieties in **14** was confirmed by the other data of its ^{13}C NMR spectrum (Table 8; Fig. 54). Indeed this latter spectrum showed the presence of five methine carbons at δ 135.3, 79.1, 69.1, 67.9, and 64.8 (C-5, C-3, C-1, C-2, and C-4, respectively); three methylenes at δ 123.8, 37.9 and 18.4 (C-8', C-4', and C-5', respectively); two methyls at δ 27.4 and 26.4 (C-9' and C-10' respectively); and one methoxy carbon at δ 59.3 (OMe). They were assigned on the basis of the couplings observed in the HSQC spectrum (Fig. 61).

On the basis of these results the structure of 1,2,4-triol-3-methoxy-6-(7-methyl-3-methylene-oct-6-ene-1-ynyl)cyclohex-5-ene was assigned phomentrioloxin (**14**).

This structure was supported by several long range couplings observed in the HMBC spectrum (Table 8; Fig. 60) and by the HR-ESIMS data (Fig. 56). Indeed, this latter spectrum showed, beside the dimeric sodium cluster at *m/z* 607, the potassium and the sodium clusters at *m/z* 331 and 315.1561, respectively.

The relative configuration of **14** was deduced from the values measured for coupling between the protons of the cyclohexene ring. On the basis of these values (Table 8), H-2 and H-3, and H-1 and H-4 were pseudo-axially and pseudo-equatorially located, while the cyclohexene ring probably assumes a twisted conformation as also observed by an inspection of a Dreiding models of **14** (Pretsch et al., 2000). This relative configuration as well as the structure assigned to **14** were confirmed by the X-ray analysis (Fig. 62).

Compound **14** crystallizes in the orthorhombic P $2_12_12_1$ space group. An ORTEP view of the molecule is shown in Figure 20. Bond lengths and angles in **14** are in the normal range (Allen, 2002). In the six-membered ring, the C-5–C-6 bond distance and the geometry around C-5 and C-6 confirmed the presence of the cyclohexene double bond. The cyclohexene ring adopts a twisted conformation with C-2 and C-3 pointing up and down from the plane formed by C-1/C-6/C-5/C-4. The ring geometry is constrained by the presence C-5–C-6 double bond. A further structural constraint is represented by a triple bond (C-1'-C-2') vicinal to the ring and a double bond between C-3' and C-8'. The two hydroxy groups at C-1 and C-4 are axial and point to opposite directions with respect to the plane of the cyclohexene ring. The third hydroxy group at C-2 is disposed in the equatorial position. The sequence of torsion angles around C3'–C4', C4'–C5', C5'–C6' bonds [-67.8(6), -71.3(6), -172.6(5), respectively] of the 7-methyl-3-methylene-oct-6-ene-1-ynyl group, generates the characteristic V-shape of the molecule. Four stereogenic centers are present in the molecule at C1/C2/C3/C4, whose relative configuration is R*/R*/R*/R*. It was not possible to determine the absolute

configuration, because of weak anomalous scattering. In the crystal packing all OH groups are involved in a pattern of intermolecular OH···O hydrogen bonds.

The relative configuration assigned to **14** was also confirmed by the couplings observed in the NOESY spectrum (Fig. 63) (Berger & Braun, 2004). Particularly significant appeared to be the correlation between H-1 and H-2, and that between H-4 and both H-5 and H-3, as well as the lack of correlation between H-2 and H-3. Finally, the correlation between the methoxy group with both Me-9' and Me-10', in agreement with the inspection of the Dreiding models of **14**, indicated a bending of the side chain towards the cyclohexene ring.
The structure assigned to phomentrioloxin was further confirmed by preparing two key derivatives (Fig. 64). By acetylation and acid-catalized ketalization, **14** was converted into 1,2,4-*O,O',O''*-triacetyl- (**17**) and 1,2-*O,O'*-isopropylidene (**18**) derivatives, respectively.

As expected, the IR spectrum of **17** (Fig. 65) lacked bands due to hydroxy groups. Its ^{1}H NMR spectrum (Fig. 66) differed from that of **14** essentially lacking hydroxy group signals, the downfield shifts (Δδ 1.46, 1.27, and 1.22) and the multiplicity of H-1, H-2, and H-4 resonating as a doublet (J = 4.1), a double doublet (J = 8.9 and 4.1 Hz) and double doublet (J = 4.4 and 4.1 Hz), at δ 5.81, 5.48, and 5.73, respectively. Finally, the presence of the three singlets at δ 2.14, 2.13, and 2.09 respectively, due to the three acetyl groups were also observed (Table 9). Its HR-ESIMS spectrum (Fig. 67) showed the sodium cluster at *m/z* 441 (see experimental).

The ^{1}H NMR data of derivative **18** (Fig. 68) differed from those of **14**, i.e., lacking the two broad singlets of the two hydroxy group at C-1 and C-2, and for the presence of two singlets of the isopropylidene group at δ 1.43 and 1.40, respectively. Furthermore, the spectrum indicate downfield shifts (Δδ 0.22 and 0.27) of the doublet (J = 5.7 Hz) and double doublet (J = 5.7 and 4.6 Hz) of H-1 and H-2 engaged in the isopropylidene ring (Table 9).

The HR-ESIMS data (Fig. 69) showed the sodium cluster of both toxin and its dimer at *m/z* 355, 687 (see experimental).

The absolute configuration of **14** was determined by applying an advanced Mosher's method (Ohtani et al., 1991). By reaction with *R*-(-)-α-methoxy-α-trifluoromethylphenylacetate (MTPA) and *S*-(+)MTPA chlorides, the 1,2-*O,O*'-isopropylidene derivative (**18**) was converted into the corresponding diastereomeric *S*-MTPA and *R*-MTPA monoesters at C-4 (**22** and **23**, respectively) (Fig. 70), whose spectroscopic data were consistent with the structure assigned to **14**. Comparison between ^{1}H NMR data (Table 10) of the *S*-MTPA (**22**) (Fig. 71) and *R*-MTPA (**23**) (Fig. 72) esters of **14** permitted assignment of the Δδ (**22-23**) values of all the protons and to assign *S*-configuration to C-4. Consequently a 1*S*, 2*S*, and 3*S* configuration was assigned to C-1, C-2 and C-3, respectively and **14** was formulated as (1*R*,2*R*,3*R*,4*R*)-3-methoxy-6-(7-methyl-3-methylene-oct-6-en-1-ynyl)-cyclohex-5-ene-1,2,4-triol.

In order to determine the relationships between the structure of phomentrioloxin and its biological properties, seven derivatives were prepared to identify the active sites of the compound and, possibly, increase or change its phytotoxicity. Together with the corresponding 1,2,4-*O,O',O"*-triacetyl derivative (**17**), by routine acetylation carried out for shorter time (5 min) with acetic anhydride and pyridine, two partially acetylated derivatives were obtained as the 1-*O*-acetyl (**15**) and 1,2-*O,O'*-diacetylphomentrioloxin (**16**) (Fig. 64). As expected, the IR spectrum of **15** and **16** (Fig. 73 and 74, respectively) still showed bands due to hydroxy groups but it also showed the very typical bands of the acetyl groups and that of the ester groups. The ^{1}H NMR spectrum of **15** (Fig. 75) differed from that of **14** essentially lacking of one hydroxy group signal, the downfield shifts (Δδ 1.35) and the multiplicity of H-1 resonating as a doublet (J = 4.1) at δ 5.70 (Table 9). The ESI MS spectrum of **15** (Fig. 76) showed the potassium and sodium clusters at *m/z* 373 and 357 (see experimental). The ^{1}H NMR spectrum of **16** (Fig. 77) differed from that of **14** essentially lacking of two hydroxy

group signal, the downfield shifts (Δδ 1.35 and 1.35) and the multiplicity of H-1 and H-2 resonating as a doublet (J = 3.8) and double doublet (J = 7.2 and 3.8) at δ 5.71 and 5.56 respectively (Table 9). The HR-ESIMS spectrum of **16** (Fig. 78) showed the potassium and sodium clusters at *m*/*z* 415 and 399 (see experimental). A different modification of the same diol system was obtained by conversion of **14** into the corresponding 1,2-*O*,*O'*-isopropylidene derivative (**18**; Fig. 64) as reported above. A further different modification of the cyclohexentriol moiety was obtained by selective oxidation to a ketone of the secondary hydroxy group C-4 with MnO_2. The ^{1}H-NMR spectrum (Fig. 79) of **19**, compared to **1**, showed the lack of the proton (H-4) of the secondary hydroxylated carbon (C-4) while the protons H-1, H-2, H-3 and H-5 resonated as a broad singlet, a double doublet (J=9.2 and 3.3 Hz), a doublet (J=9.2) and a singlet at δ 4.56, 4.05, 4.00 and 6.21 respectively (Table 9). The IR spectrum (Fig. 80) of **19**, compared to **14** showed an α-β unsaturated ketone group. The HR-ESIMS spectrum of **19** (Fig. 81) showed the sodium cluster at *m*/*z* 313 (see experimental). The derivative **19** (Fig. 64) also showed a marked modification of the stereochemistry of the cyclohexene ring. Several attempts were made using different catalysts, solvents and time of reaction to partially or totally hydrogenate the triple and the two double bond of the side chain at C-6. These reactions aimed at modifying differently the 7-methyl-3-methylene-oct-6-ene-1-ynyl and at having responses on the role of its functionalities on the biological activity. The best results were obtained using 5% Rd/C in MeOH at atmosphere pressure and at room temperature. The main derivative obtained was the 1',1',2',2',3',8',6',7'-octahydroderivative (**20;** Fig. 64), as the other partially hydrogenated compounds were present in very low yield in the reaction mixture. **20** showed the complete saturation of the side chain at C-6 that is now a 3,7-dimethyloctyl. The ^{1}H-NMR spectrum (Fig. 82) showed the appearance of complex multiplets integrating for 12 protons in the region of δ 2.00-1.00, while the protons H-8', H-9' and H-10' were overlapped and resonated as doublets having the same J (6.5), at δ 0.90 (Table 9). The IR spectrum (Fig. 83), compared

to that of **14**, differed essentially for the lack of the olefinic bands. The HR-ESIMS spectrum of **20 16** (Fig. 84) showed the potassium and sodium clusters at *m*/*z* 339 and 323 (see experimental). Finally the reductive opening of the cyclohexentriol moiety was obtained by the selective oxidation cleavage with $NaIO_4$ of the diol system present between C-1 and C-2. The corresponding unstable dialdehyde was immediately reduced with $NaBH_4$ to the corresponding primary hydroxy groups yielding the derivative **21**. The latter being the 2-methoxy-5-(3-methylene-oct-6-en-1-ynyl)-hex-4-ene-1,3,6-triol (**21**; Fig. 64) showed actually a structure completely different to the parent compound, except for the part corresponding to the side chain. Its ^{1}H NMR spectrum (Fig. 85) differed from that of **14** for the lacking of hydroxy group signals, for the presence of two broad doublets at δ 4.28 and 4.19 due to H_2 C-1 and two doublets at δ 3.82 and 3.77 due to H_2 C-2, for the downfield shifts (Δδ 0.41 and 0.15) and the multiplicity of H-3 resonating as a multiplet at δ 3.29 (Table 9)

. The HR-ESIMS spectrum of **21** (Fig. 86) showed the sodium cluster at *m*/*z* 317 (see experimental).

5.4. Biological activity of phomentrioloxin and its derivatives

A broad number of different biological assays were performed with the main toxin and the seven derivatives. Assayed on leaves of several weeds at 40 µg/droplet (6.85 mM), phomentrioloxin (**14**) caused the appearance of necrotic lesions one day after the application. Large necrosis (around 1 cm diameter) were particularly evident on the leaves of *Cirsium arvense* and *Sonchus oleraceus*. Necrosis slightly smaller (6-8 mm) were clearly observable on leaves of the host plant *C. lanatus*, but also of the other two dicotyledonous weed species tested, i.e.: *Mercurialis annua* and *Chenopodium album*. Necrotic spots were smaller (diameter around 3 mm) on *Setaria viridis* (monocot). Assayed at 3.42 mM, lesions were still clearly observable on *C. arvense*, *S. oleraceus*, and the host as well, whereas at 1.71 mM, it was active only on *C. arvense* leaves.

Assayed on leaves by puncture, **15** proved to be as toxic as **14**, causing the appearance of wide necrosis (Table 11); compounds **18** and **20** caused the appearance of necrosis of modest size, whereas all the other compounds proved to be inactive. On protoplasts, together with **14 (**60% protoplast viable), only **15** and **20** proved to be active, leaving 42 and 48 % protoplasts viable after the treatment, respectively (viability of the control 85%). All the other compounds were ineffective at the concentration tested.

The results showed that the hydroxy groups at C-2 and C-4 are very important features for the phytotoxicity. In fact, when the HO-2 as in **16** or both HO-2 and HO-4 were acetylated as in **17** the total loss of activity was observed. The role of these two hydroxy groups (HO-2 and HO-4) was confirmed by the reduced activity observed when the hydroxy group at C-2, together with that at C-1, was ketalized as in **18,** and by the total loss of the activity when the hydroxy group at C-4 was oxidized as in **19**. The hydroxy group at C-1 seems not to be important for the activity. In fact, when it was acetylated as in **15**, this derivative proved to be as toxic as **14**. The reduced activity also showed by derivative **20** indicated that the unsaturation of the geranyl side chain also plays a role into impart the phytotoxicity. Finally, the lack of activity of derivative **21** also meant that the presence of an unchanged cyclohexenetriol ring is important for the activity.

When assayed on brine shrimps at 1.7×10^{-4} M, only compound **15** caused 45% larvae mortality after 24 hours exposure to the toxin, which increased to 84% at 48 hours. All the other compounds were inactive at the same concentration (Table 11). The activity of derivative **15** could indicate that it can more easily penetrate the cell membrane, due to his increased lipophilicity. Probably the conversion of the acetyl derivative **15** into the parent compound occurs at physiological pH as frequently observed in other natural metabolites, and is known as “lethal metabolism” (Hassal, 1990). This may not occur completely for the analogues diacetyl and triacetyl derivatives **16** and **17.**

None of the compounds was toxic when assayed on Gram+, Gram- bacteria, and *G. candidum* using concentrations up to 60 µg/diskette (Table 11).

These results could be useful when studying the mode of action of phomentrioloxin (**14**) and in devising new natural products with potential application as herbicides in agriculture.

6. CONCLUSIONS

1) From the liquid culture of *A. agropyrina*, a fungal pathogen of *Elytrigia repens*, three new coumpound, named agropyrenol, agropyrenale and agropyrenone, were isolated and characterized. Their structure was established by spectroscopic and chemical methods and their biological activity was studied. When assayed on different weedy plants agropyrenol showed a strong phytotoxic activity, agropyrenale was less active, while agropyrenone was inactive. None of compounds showed antibiotic, fungicide or zootoxic activity.

2) Six derivatives obtained by chemical modifications of agropyrenol were assayed for phytotoxic antifungal, antibiotic and zootoxic activities, and a structure-activity relationship was examined. Both the double bond and the diol system of the 3,4-dihydroxypentenyl side chain, as well as the aldehyde group at C-6 and C-1 of the phenolic ring of agropyrenol proved to be important for the phytotoxicity. The lesser polar 3',4'-*O,O'*-isopropyledene of agropyrenol showed also a significant zootoxic and a slight antimicrobial activity. This finding could be useful in devising new natural herbicides for practical application in agriculture.

3) From the liquid culture of *D. gulyae*, phytopathogenic fungi isolated from *C. lanatus*, a new phytotoxic geranylcyclohexentriol, named phomentrioloxin, was isolated. The structure of phomentrioloxin was established by spectroscopic, X-ray, and chemical methods and its biological activity was studied. When assayed on different weedy plants phomentrioloxin showed a strong phytotoxic activity. It was inactive to antibiotic, antifungal and zootoxic assays.

4) Seven derivatives obtained by chemical modifications of phomentrioloxin were assayed for phytotoxic, antifungal, antibiotic and zootoxic activities, and a structure-activity relationship was examined. The hydroxy groups at C-2 and C-4 appeared to be important features for the phytotoxicity, as well as an unchanged cyclohexentriol ring. A role seemed also to be played by the unsaturations of the geranyl side chain. These findings could be

useful for understanding the mechanisms of action of novel natural products, for identifying the active sites, and possibly in devising new herbicides of natural origin.

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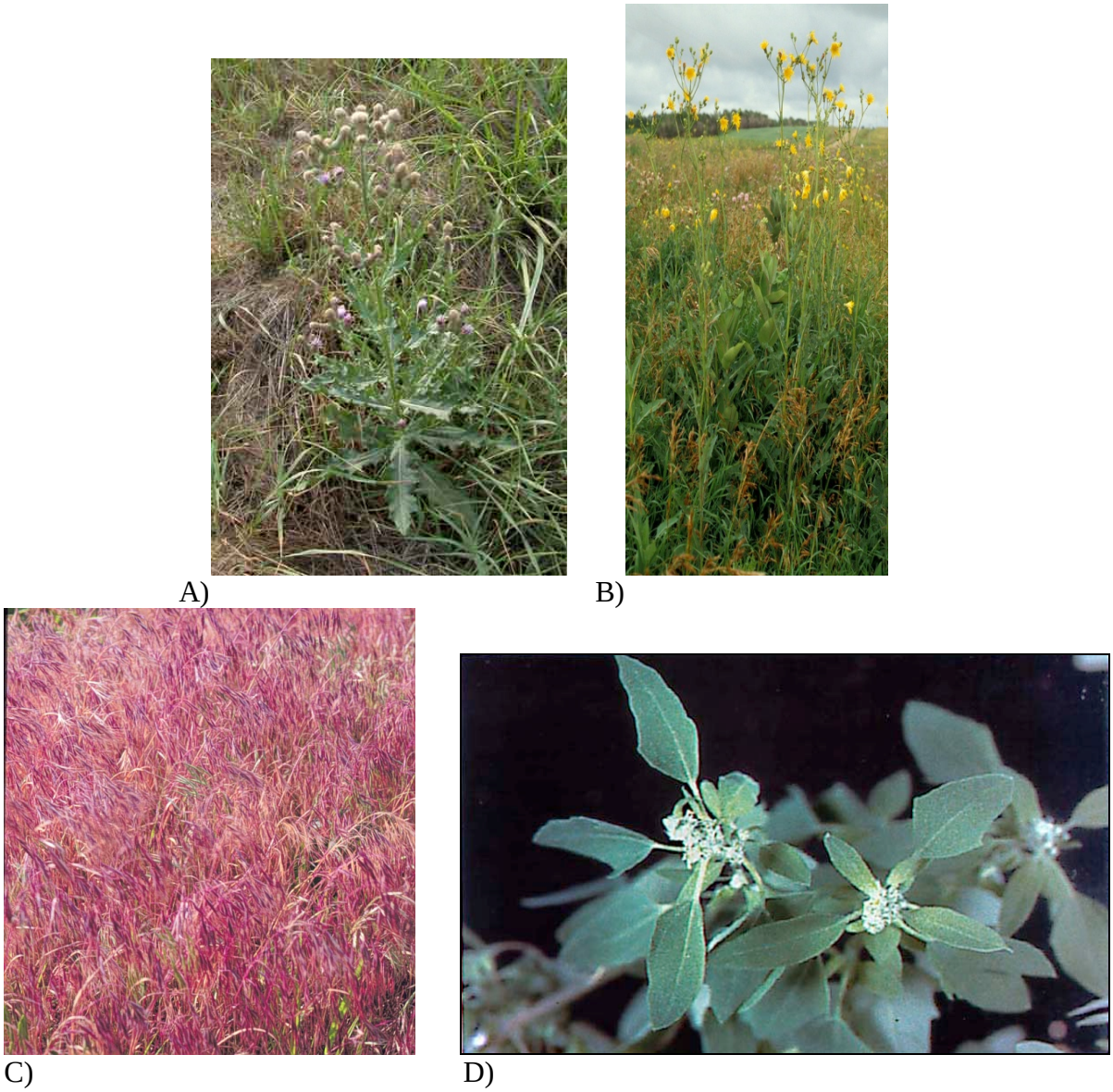
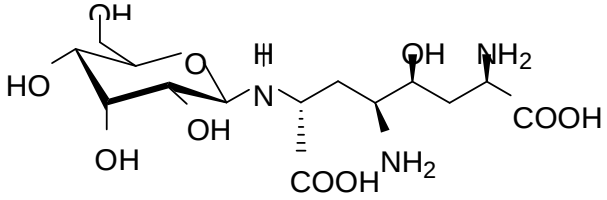


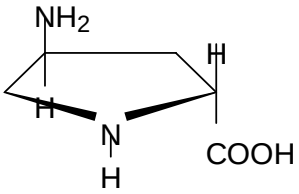
A) B)

C) D)

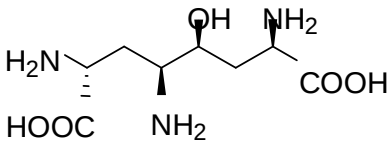
Fig. 1. Weed species: A) *C. arvense;* B) *Sonchus arvensis;* C) *Bromus tectorum;* D) *Chenopodium album*



Ascaulitoxin

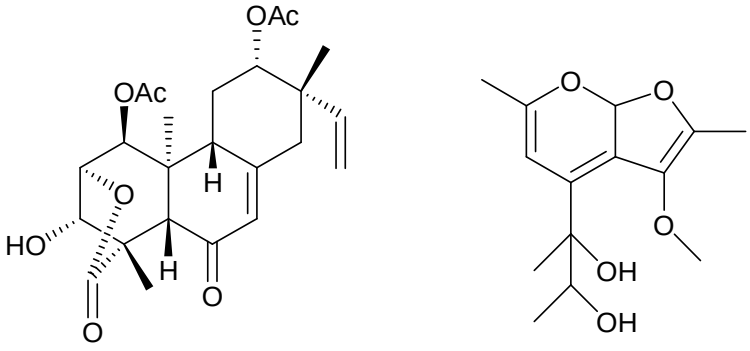


4-Ammino-D-proline



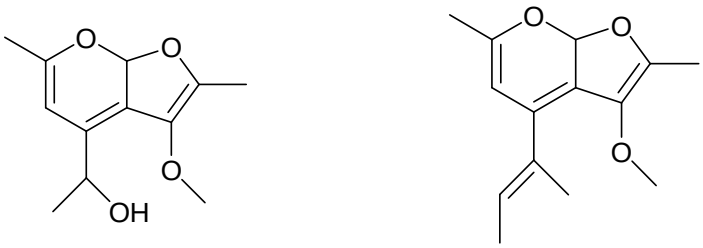
Ascaulitoxin aglycone

Fig. 2. Phytotoxins produced by *Ascochyta caulina*



Chenopodoline

Chenopodolan A



Chenopodolan B

Chenopodolan C

Fig. 3. Metabolites produced by *Phoma chenopodiicola*

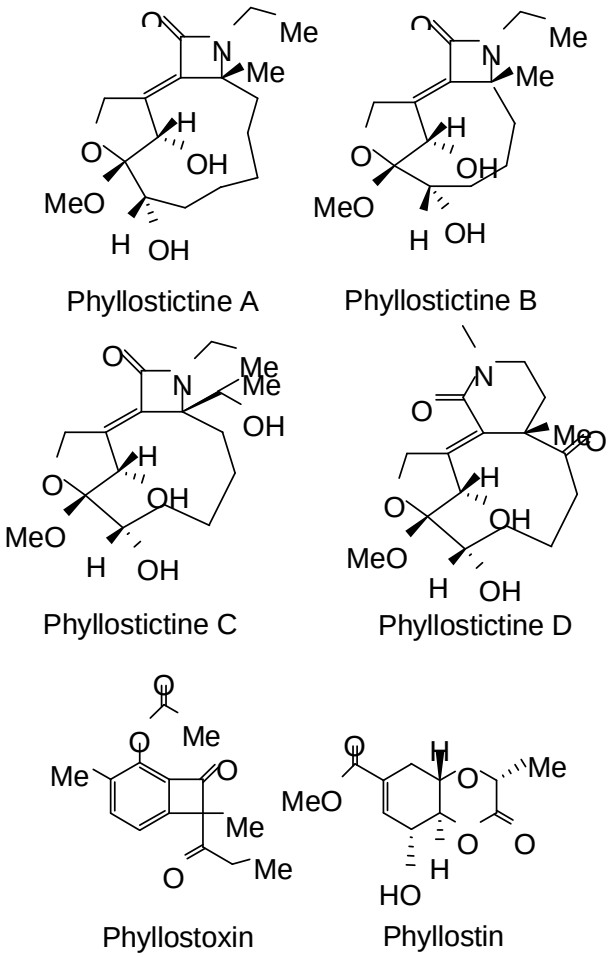


Fig. 4. Metabolites produced by *Phyllostictica cirsii*

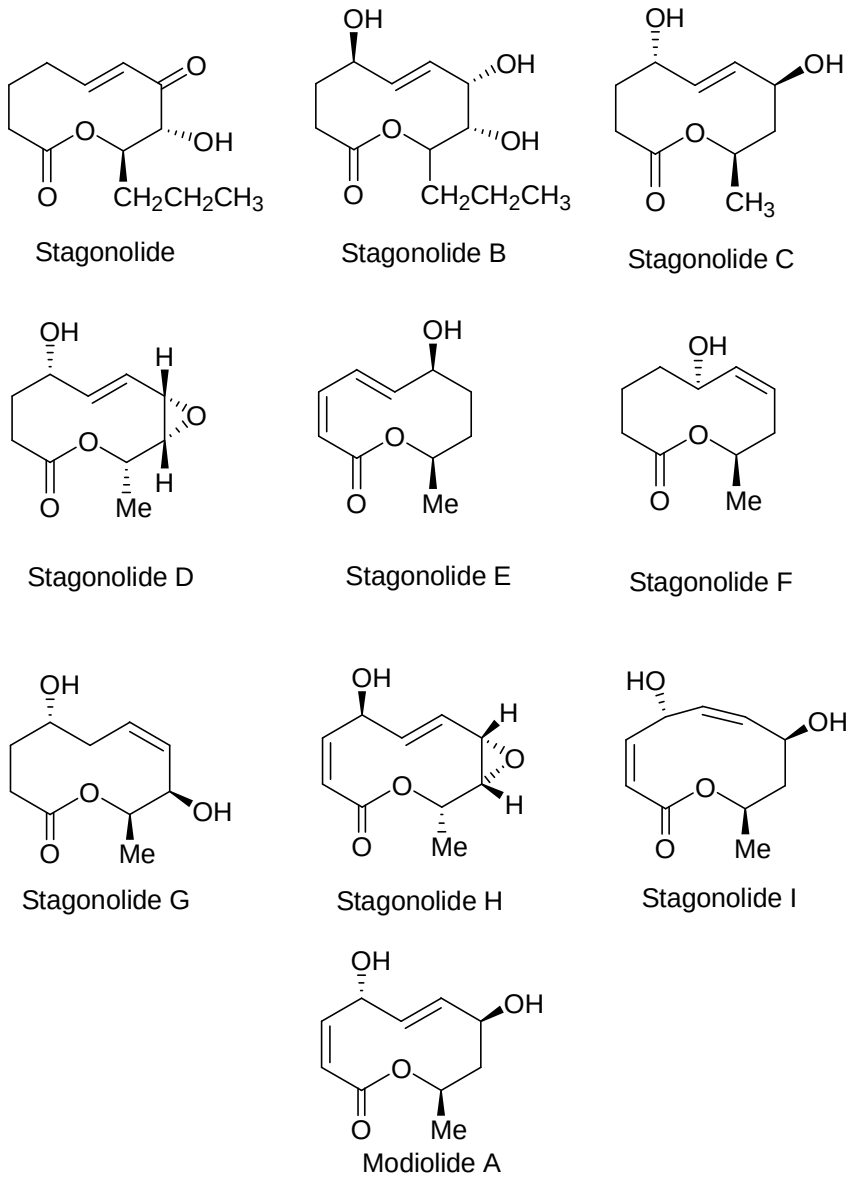
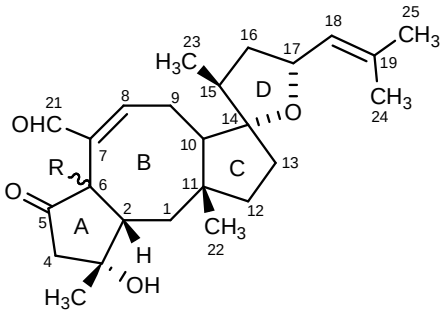
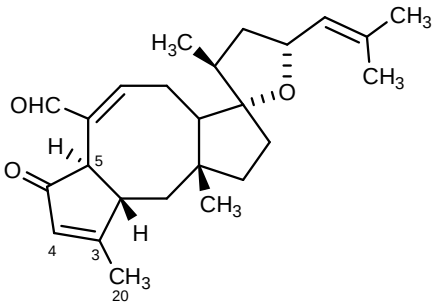


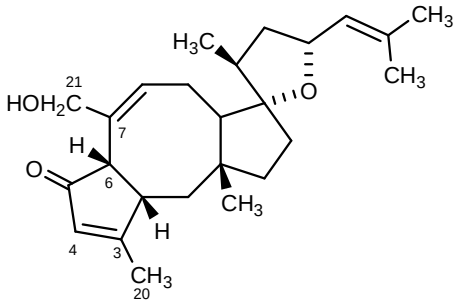
Fig. 5. Stagonolides produced by *Stagonospora cirsii*



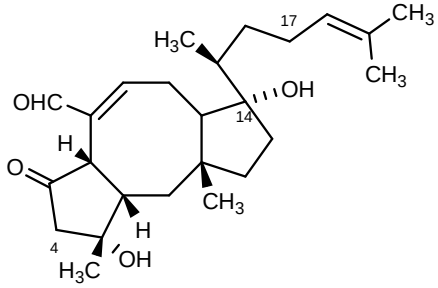
Ophiobolin A R=bH
6-*Epi*-ophiobolin A R=aH



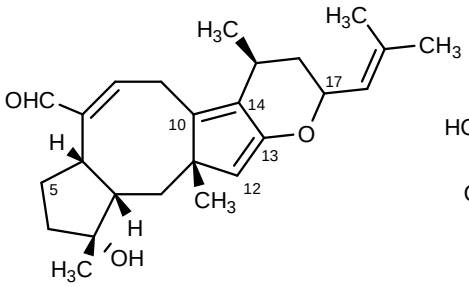
3-Anidro-6-*epi*-ophiobolin A



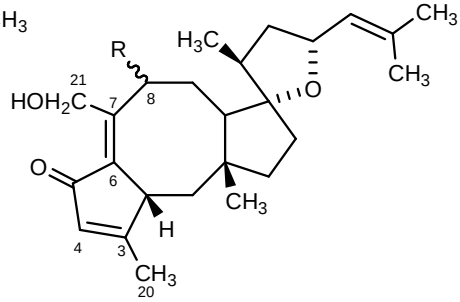
Ophiobolin I



Ophiobolin B

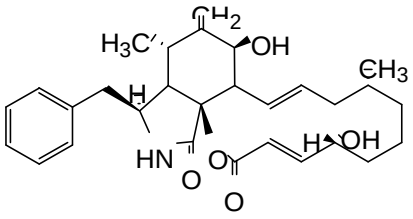


Ophiobolin E

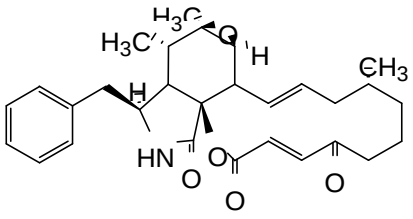


Ophiobolin J R=$^{\beta}$OH
8-*Epi*-ophiobolin J R=$^{\alpha}$OH

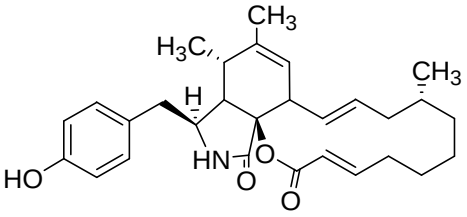
Fig. 6. Ophiobolins produced by *Drechslera gigantea*



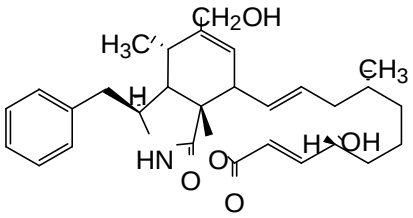
Cytochalasin B



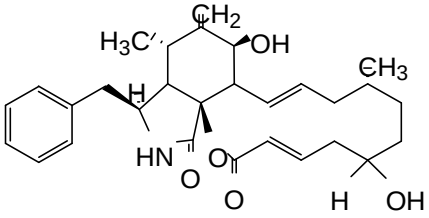
Cytochalasin F



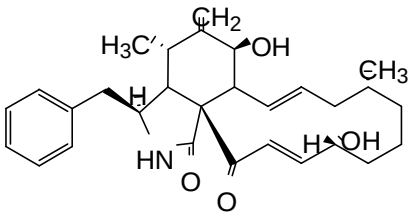
Cytochalasin Z1



Cytochalasin Z2

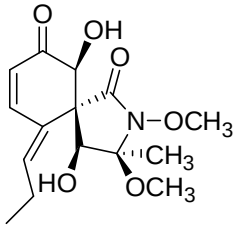


Cytochalasin Z3

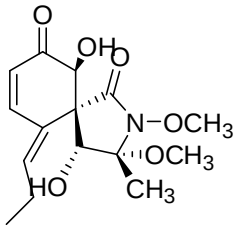


Deoxaphomin

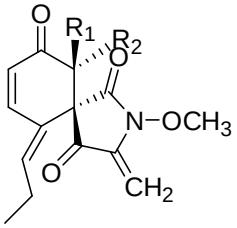
Fig. 7. Cytochalasins isolated from liquid and solid cultures of different strains of *Phoma exigua* var. *exigua* and *Pyrenophora seminiperda* in solid culture



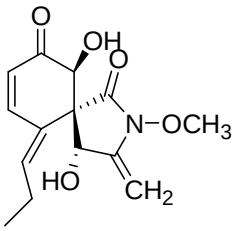
Spirostaphylotrichin W



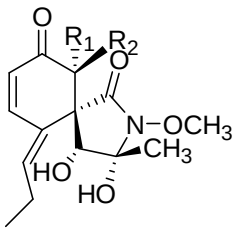
Spirostaphylotrichin V



Spirostaphylotrichin C R_1=OH R_2=H
Spirostaphylotrichin D R_1=H R_2=OH

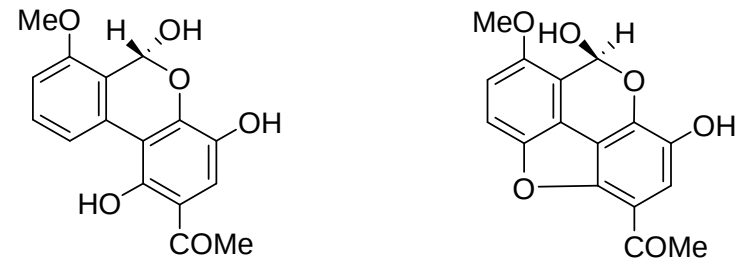


Spirostaphylotrichin A



Spirostaphylotrichin R R_1=OH R_2=H
Triticone R_1=H R_2=OH

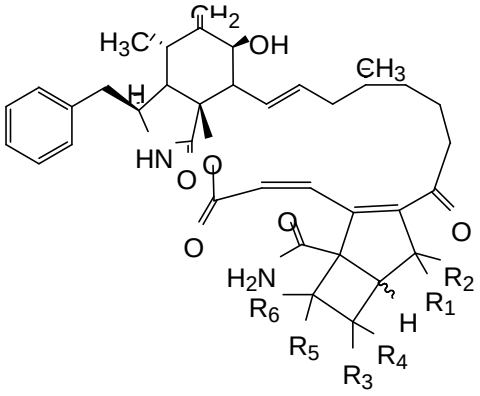
Fig. 8. Metabolites produced by *Pyrenophora seminiperda* in liquid culture

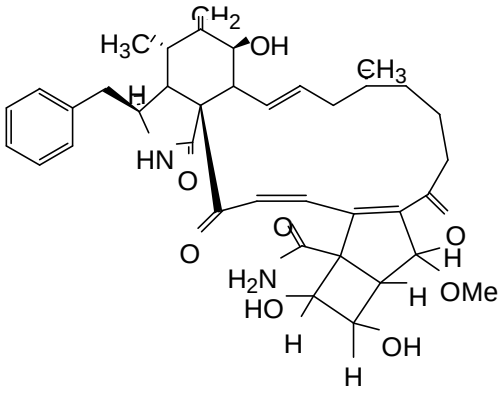


Alternethanoxin A

Alternethanoxin B

Fig. 9. Alternethanoxins produced by *Alternaria sonchi*





Phomachalasins A R_1=OCH_3, R_2=R_3=R_5=H, R_4=R_6=OH

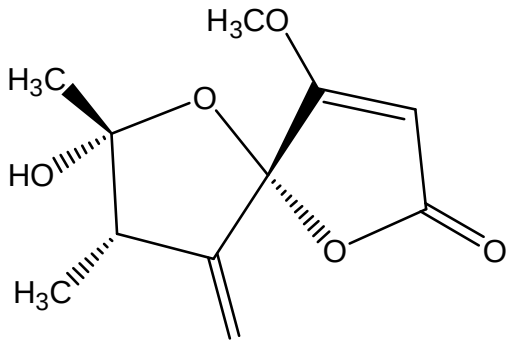
Phomachalasins C R_1=OCH_3, R_2=R_4=R_6=H, R_3=R_5=OH

Phomachalasins D R_1=OCH_3, R_2= R_3=R_6=H, R_4=R_5=OH

Phomachalasin B

Fig. 10. Phomachalasins produced by *Phoma exigua* var. *exigua*

Fig. 11. *Elytrigia repens* (top) and inflorescence (below)



Papyracillic acid

Fig. 12. Structure of papyracillic acid isolated from solid culture of *A. agropyrina* var. *nana*

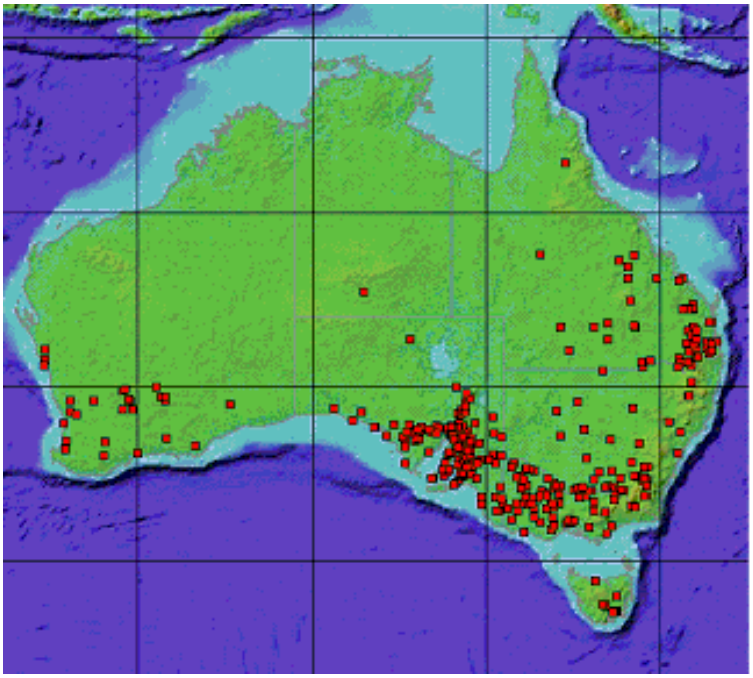


Fig. 13. Distribution of saffron thistle in Australia

Fig. 14. Saffron thistle and its flowering

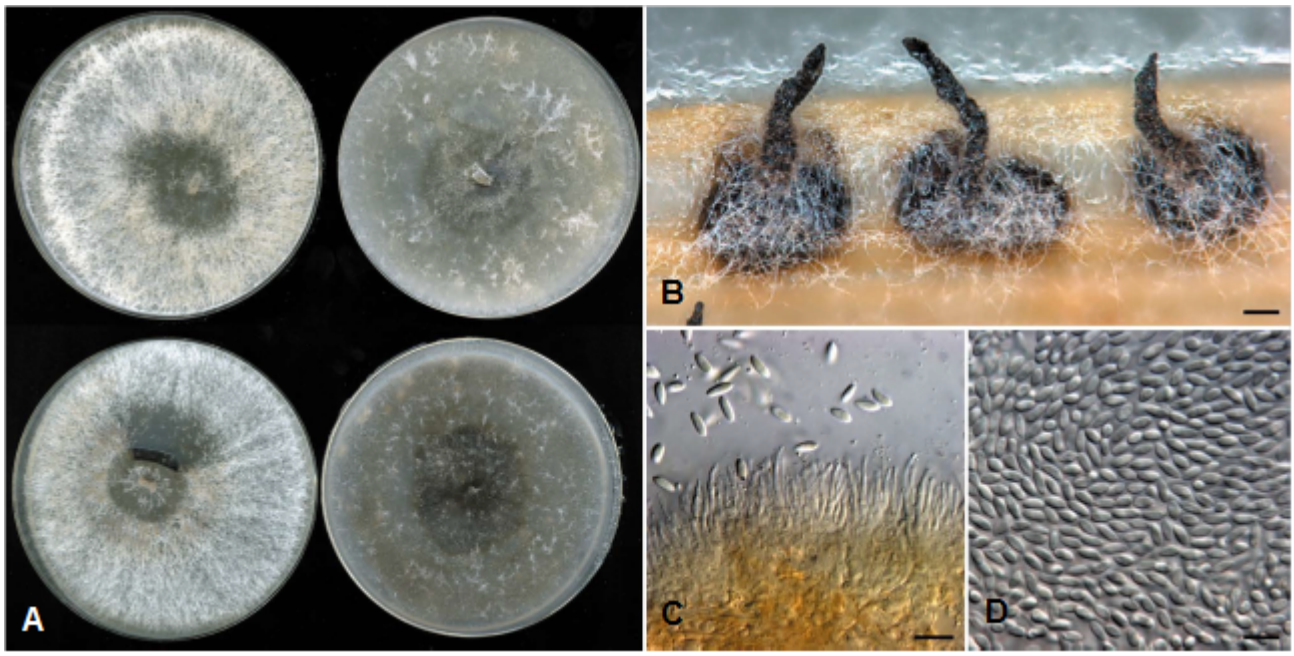


Fig. 15. *Diaporthe gulyae.* A) Cultures on PDA (left), OA (right) after 7 days (top) and 28 d (bottom); B) pycnidial beaks on sterilised wheat straw; C) conidia and conidiophores; D) alpha conidia. Scale bars: b = 100 µm; c, d = 10 µm

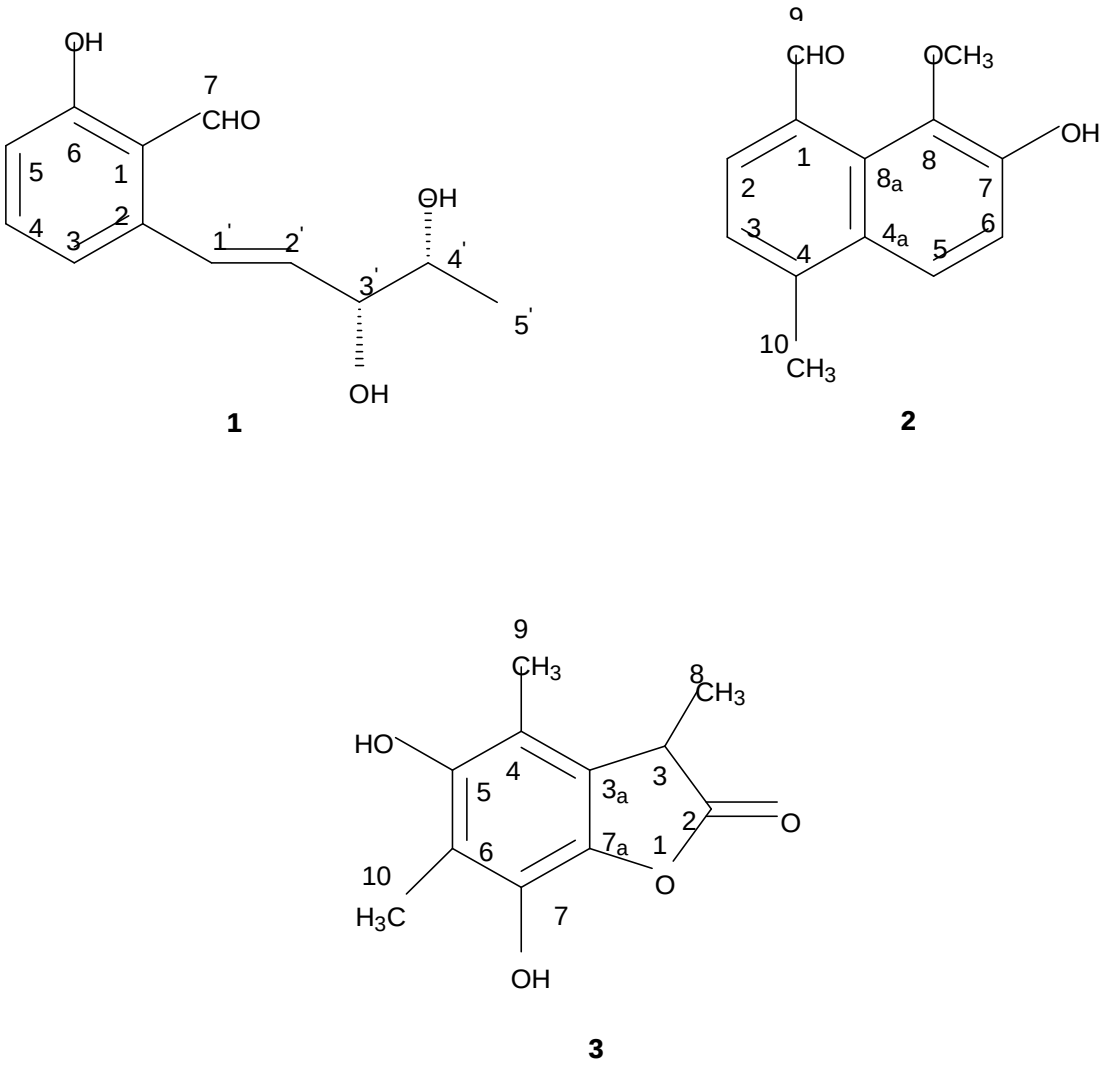


Fig. 16. Structure of agropyrenol (**1**), agropyrenal (**2**) and agropyrenone (**3**) isolated from *Ascochyta agropyrina* var. *nana*

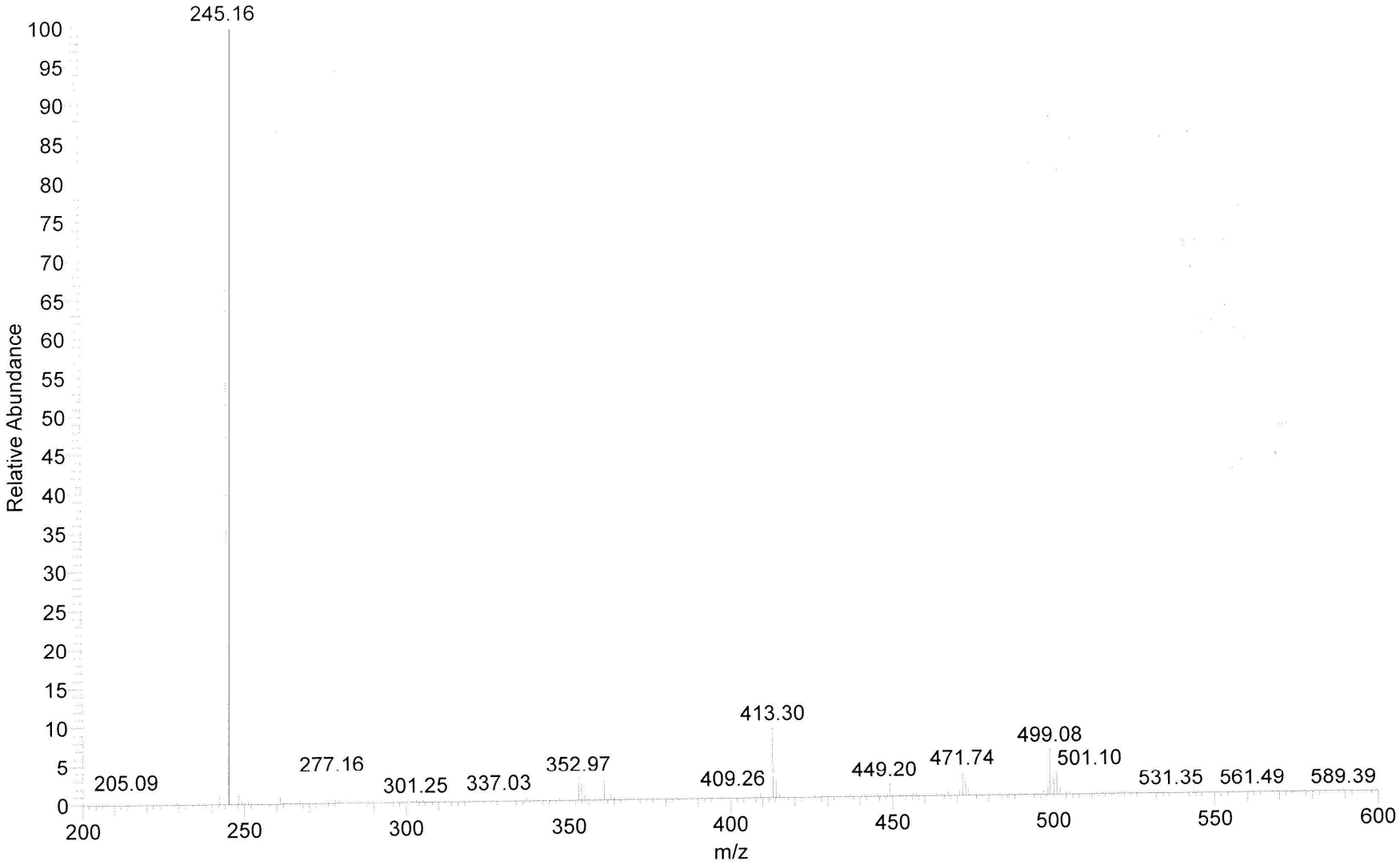


Figure 17. ESI MS spectrum of agropyrenol (**1**) recorded in positive modality.

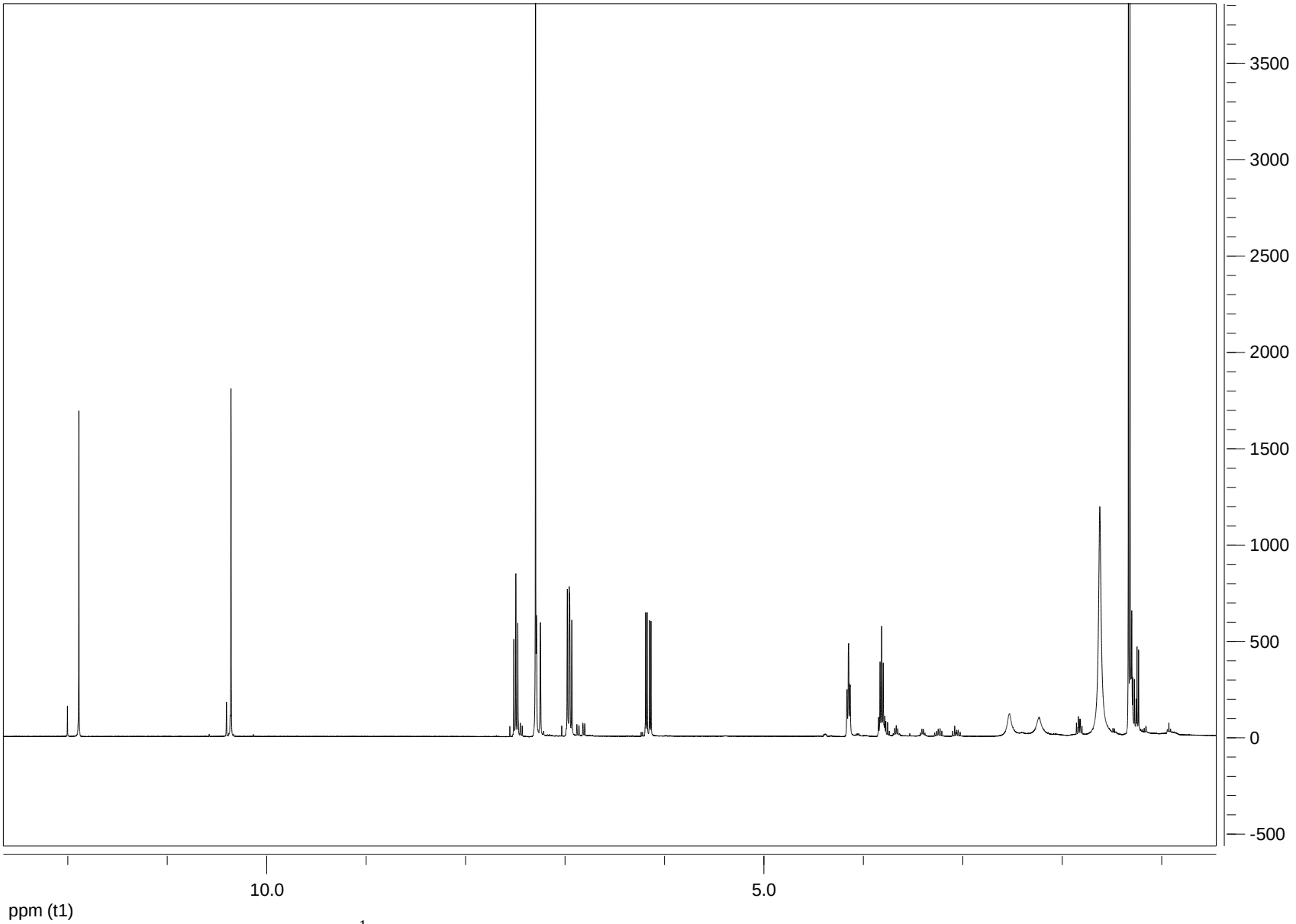


Figure 18. ^{1}H NMR spectrum of agropyrenol (**1**) recorded in $CDCl_3$ at 400 MHz.

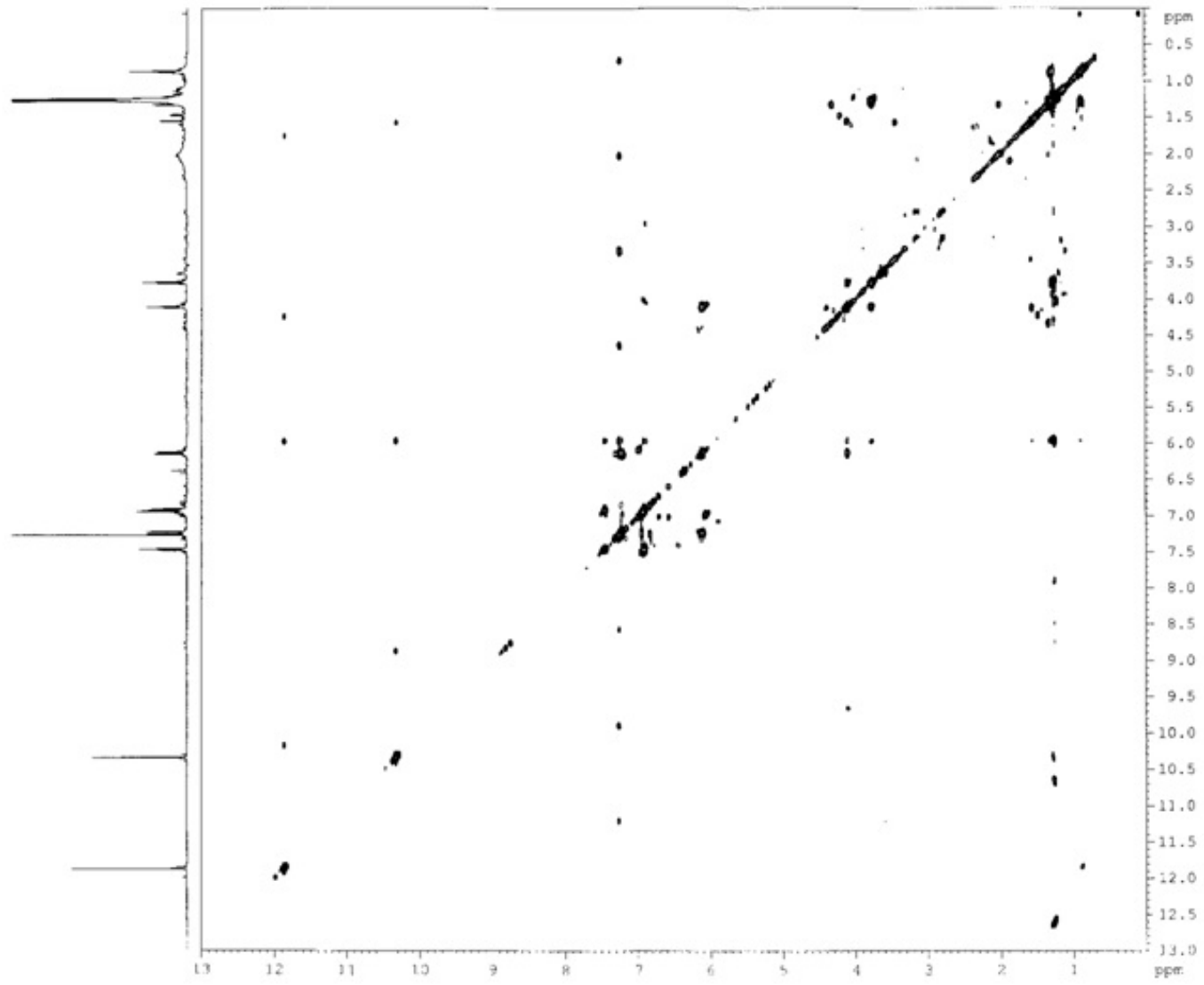


Figure 19. COSY spectrum of agropyrenol (**1**) recorded in $CDCl_3$ at 400 MHz.

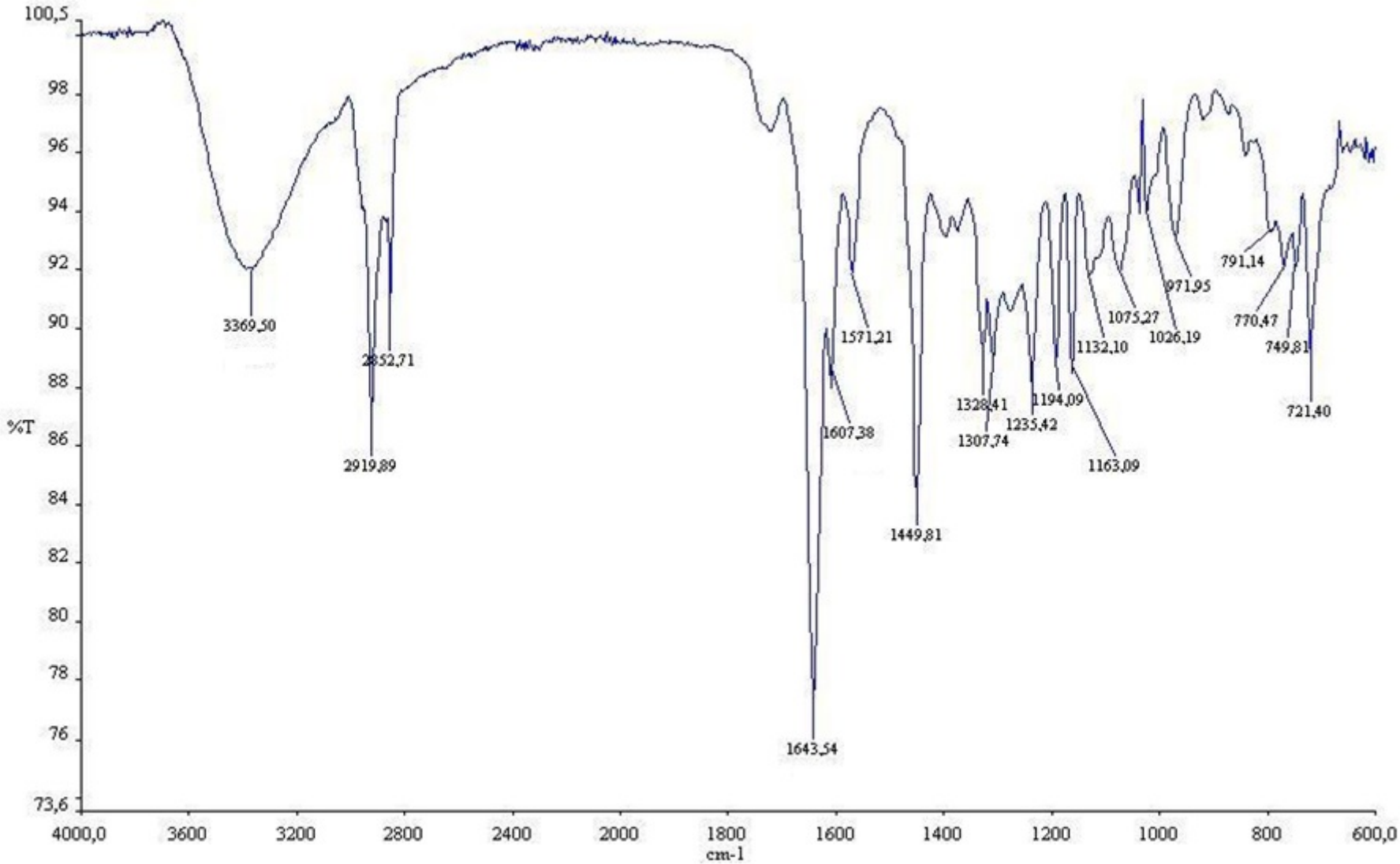


Figure 20. IR spectrum of agropyrenol (**1**) recorded as glass liquid.

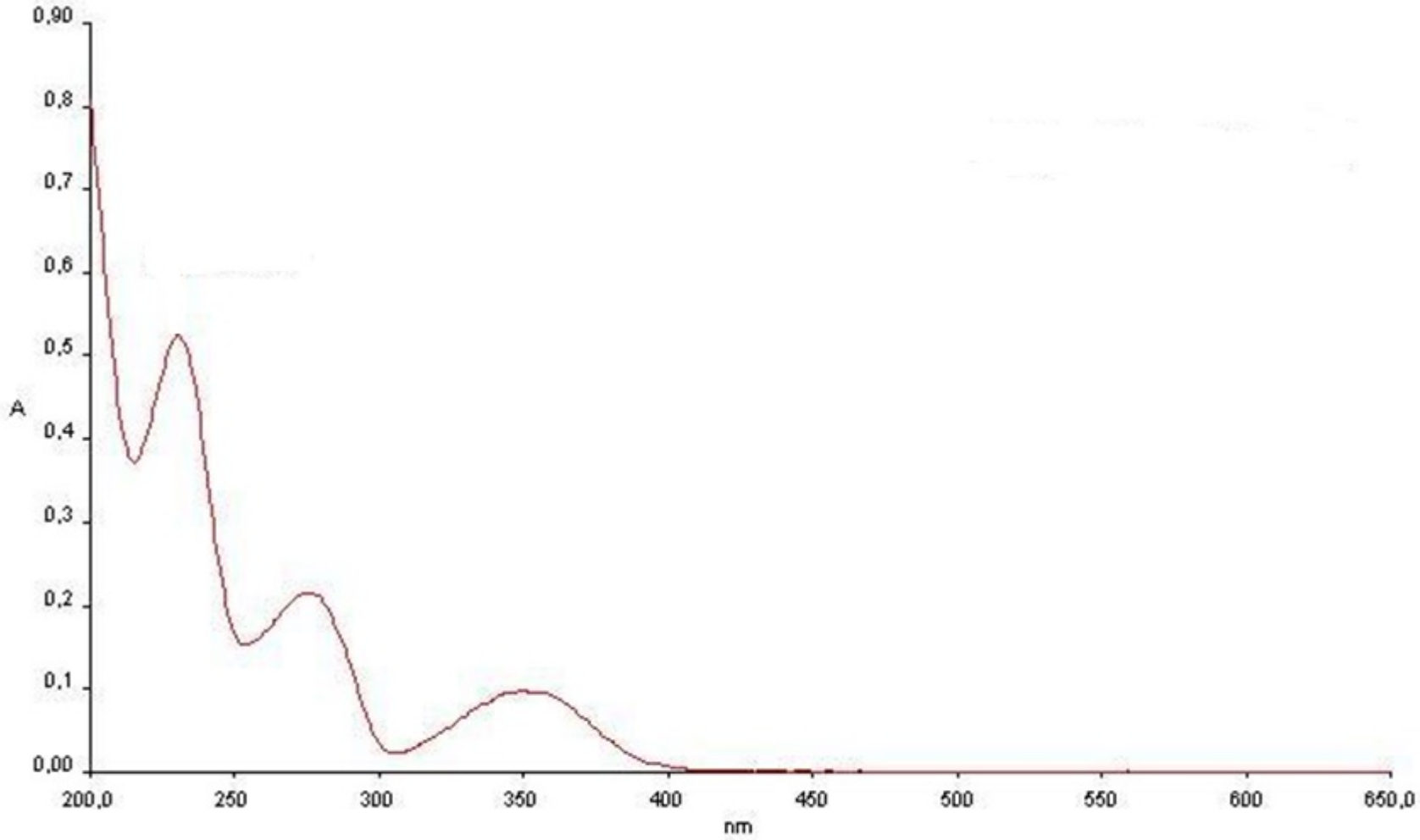


Figure 21. UV spectrum of agropyrenol (**1**) recorded in MeCN solution.

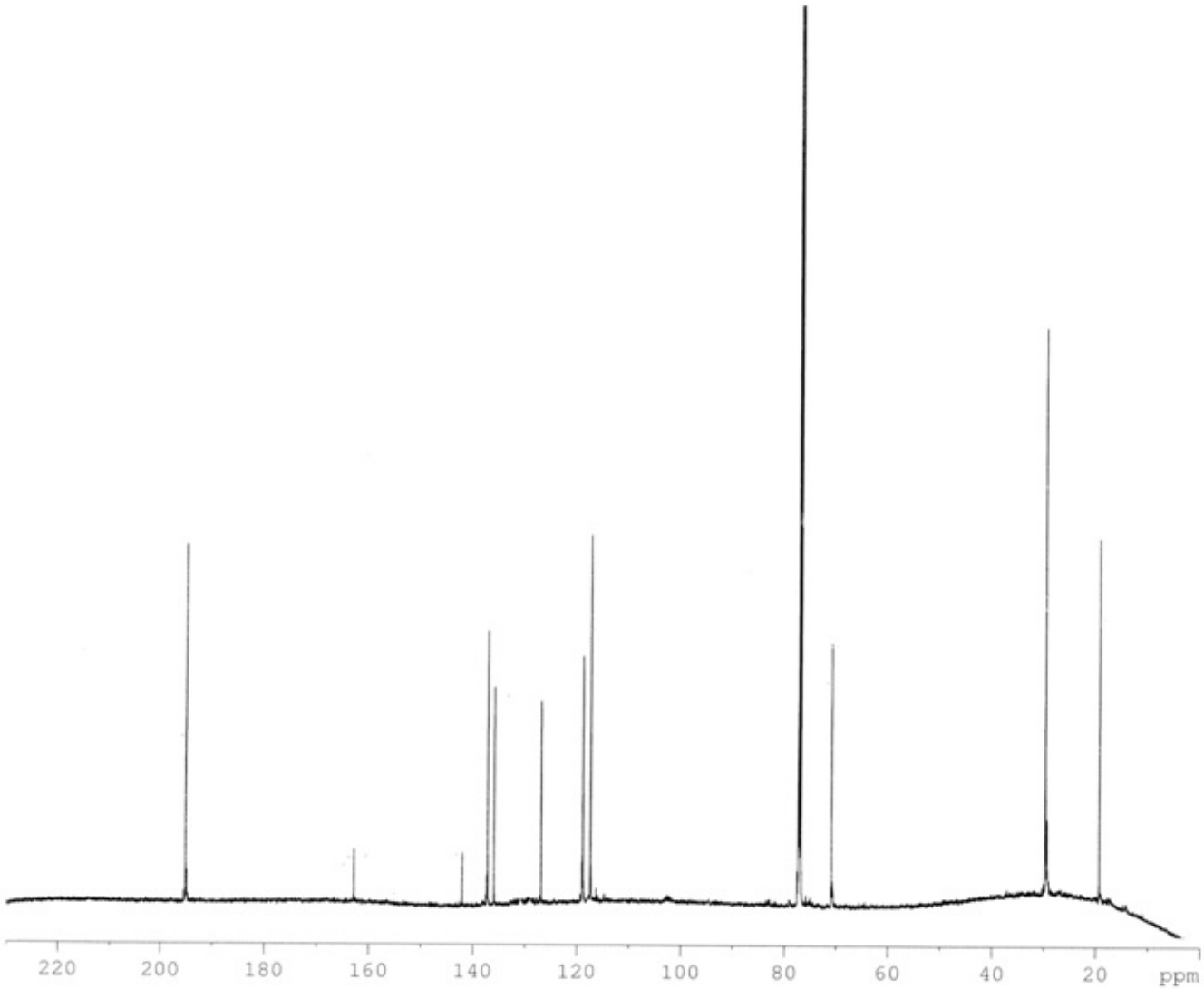


Figure 22. ^{13}C NMR spectrum of agropyrenol (**1**) recorded in $CDCl_3$ at 400 MHz.

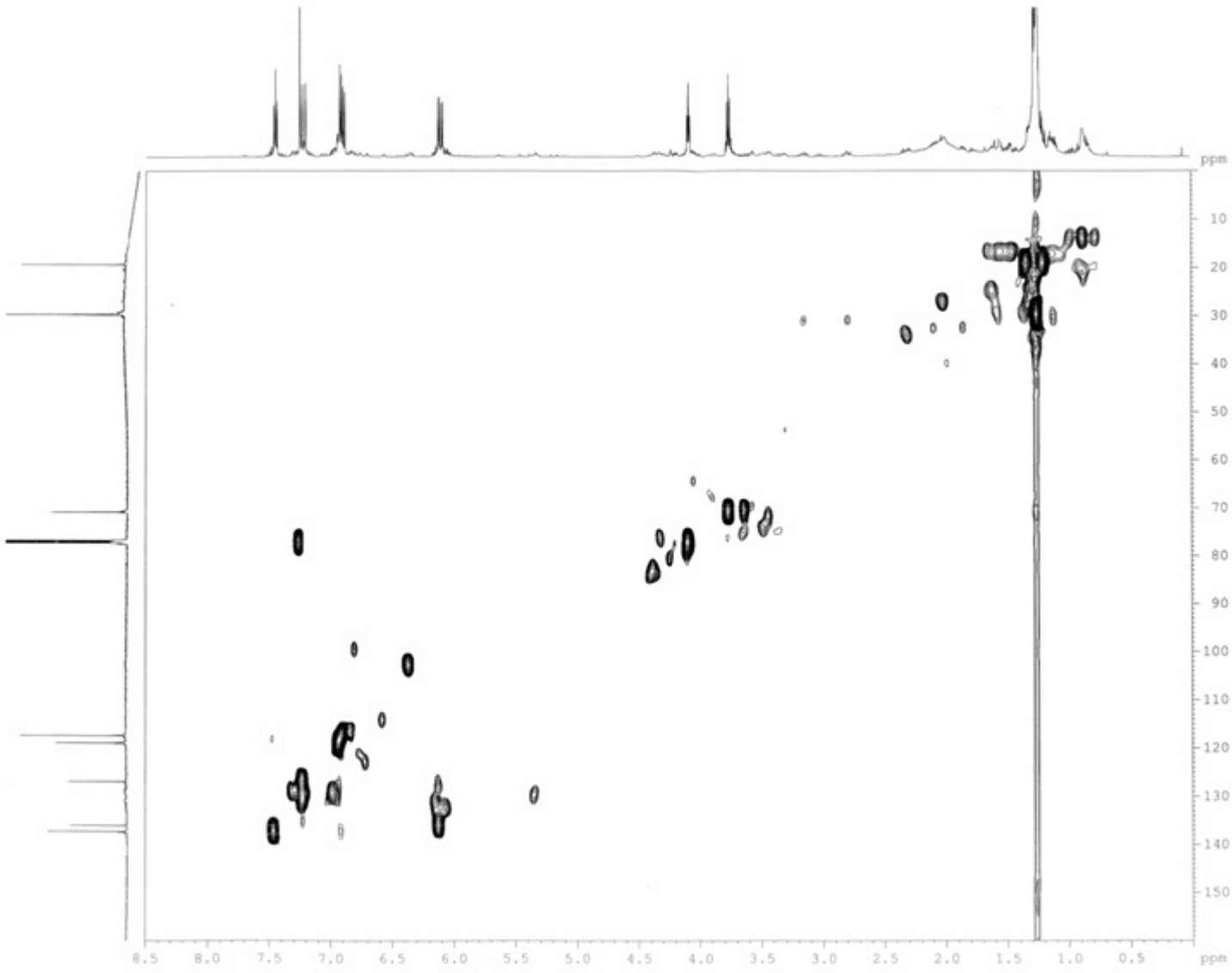


Figure 23. HSQC spectrum of agropyrenol (**1**) recorded in $CDCl_3$ at 400 MHz.

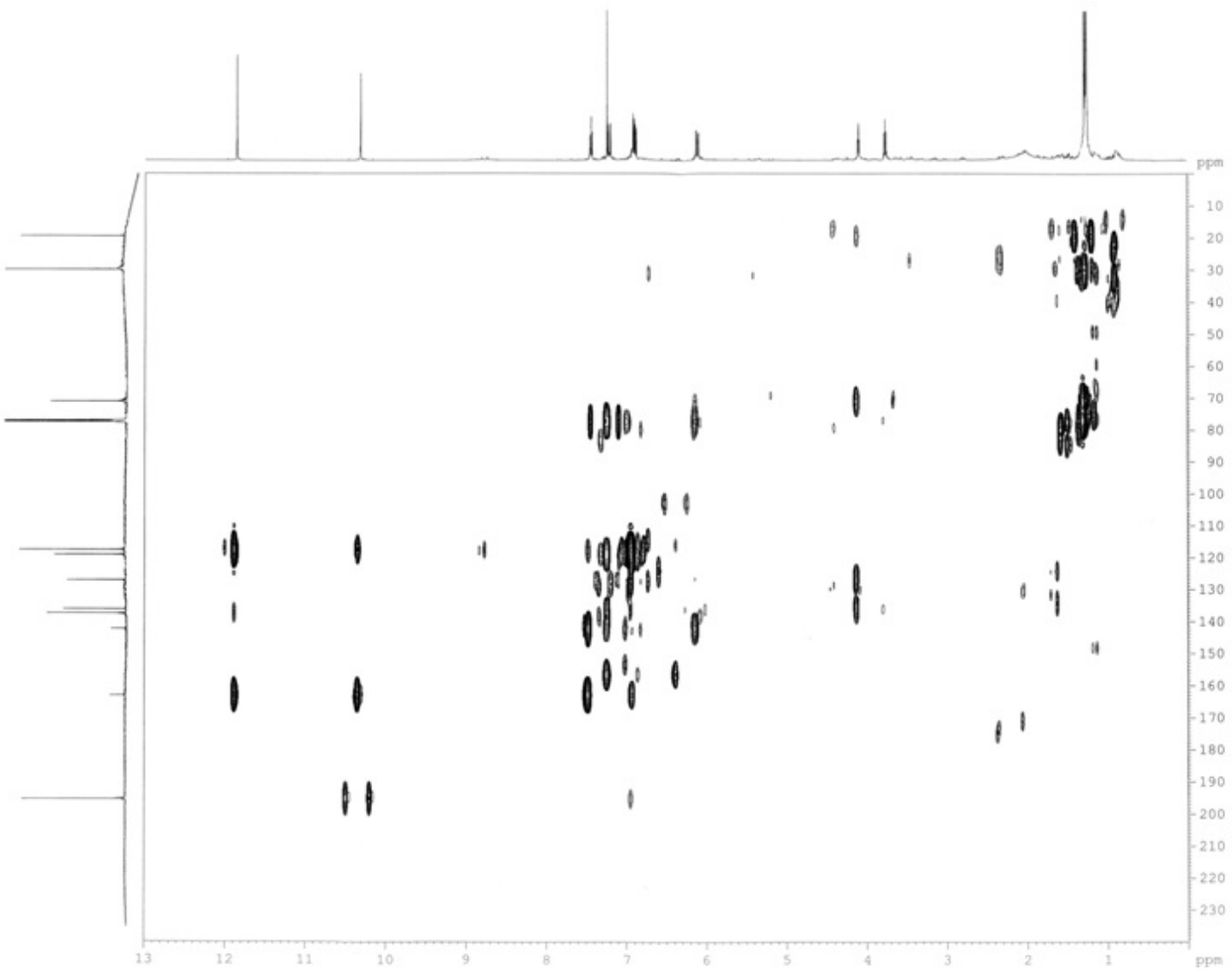


Figure 24. HMBC spectrum of agropyrenol (**1**) recorded in $CDCl_3$ at 400 MHz.

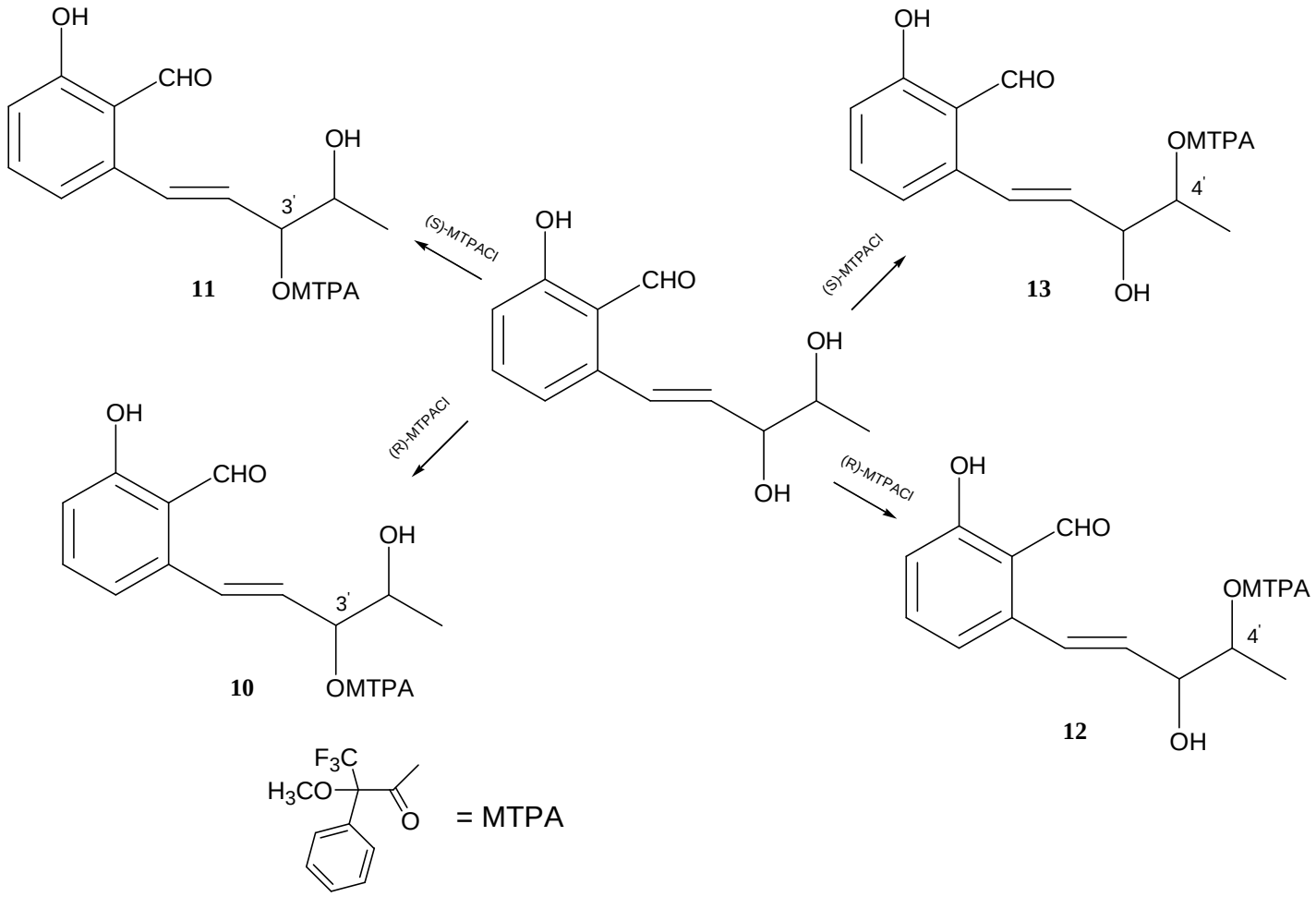


Figure 25. Preparation of *S*-3', *R*-3' and *S*-4', *R*-4'-α-methoxy-α-trifluorophenylacetate (MTPA) of agropyrenol (**10**, **11**, **12** and **13**, respectively)

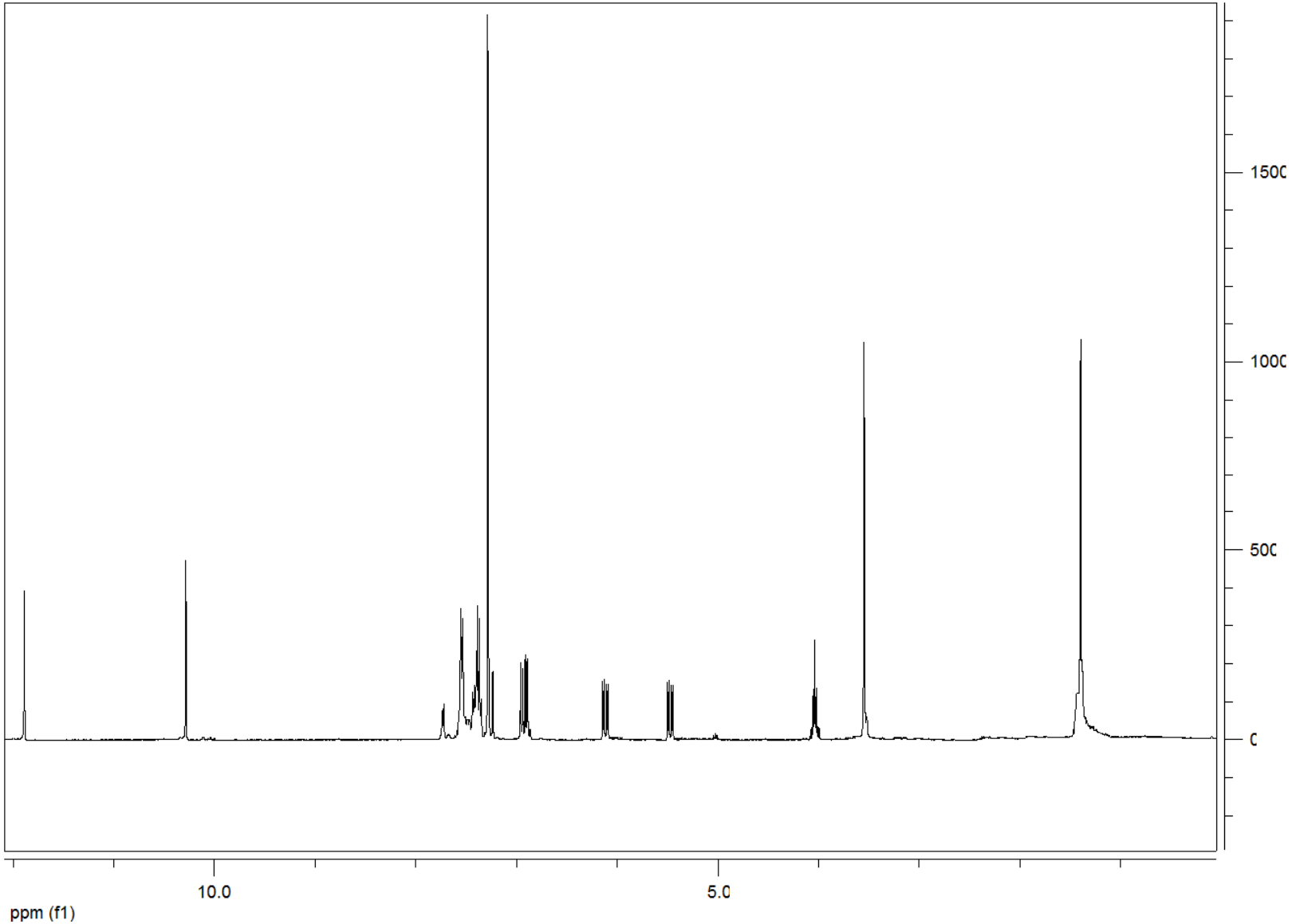


Figure 26. ^{1}H NMR spectrum of 3'-*O*-(*S*)-α-methoxy-α-trifluoromethyl-α-phenylacetate (MTPA) ester of agropyrenol (**10**) recorded in $CDCl_3$ at 400 MHz.

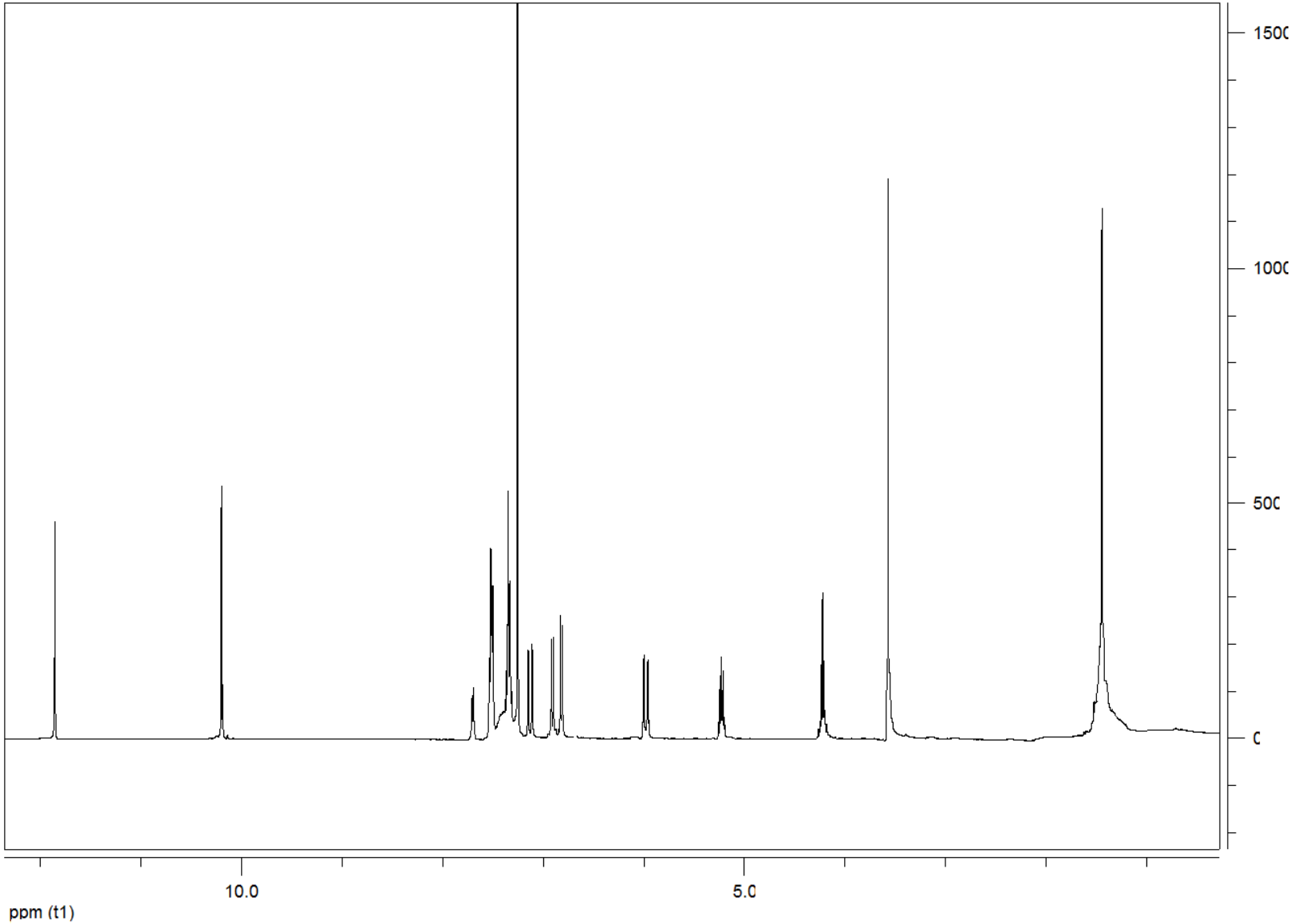


Figure 27. ^{1}H NMR spectrum of 3'-*O*-(*R*)-α-methoxy-α-trifluoromethyl-α-phenylacetate (MTPA) ester of agropyrenol (**11**) recorded in $CDCl_3$ at 400 MHz.

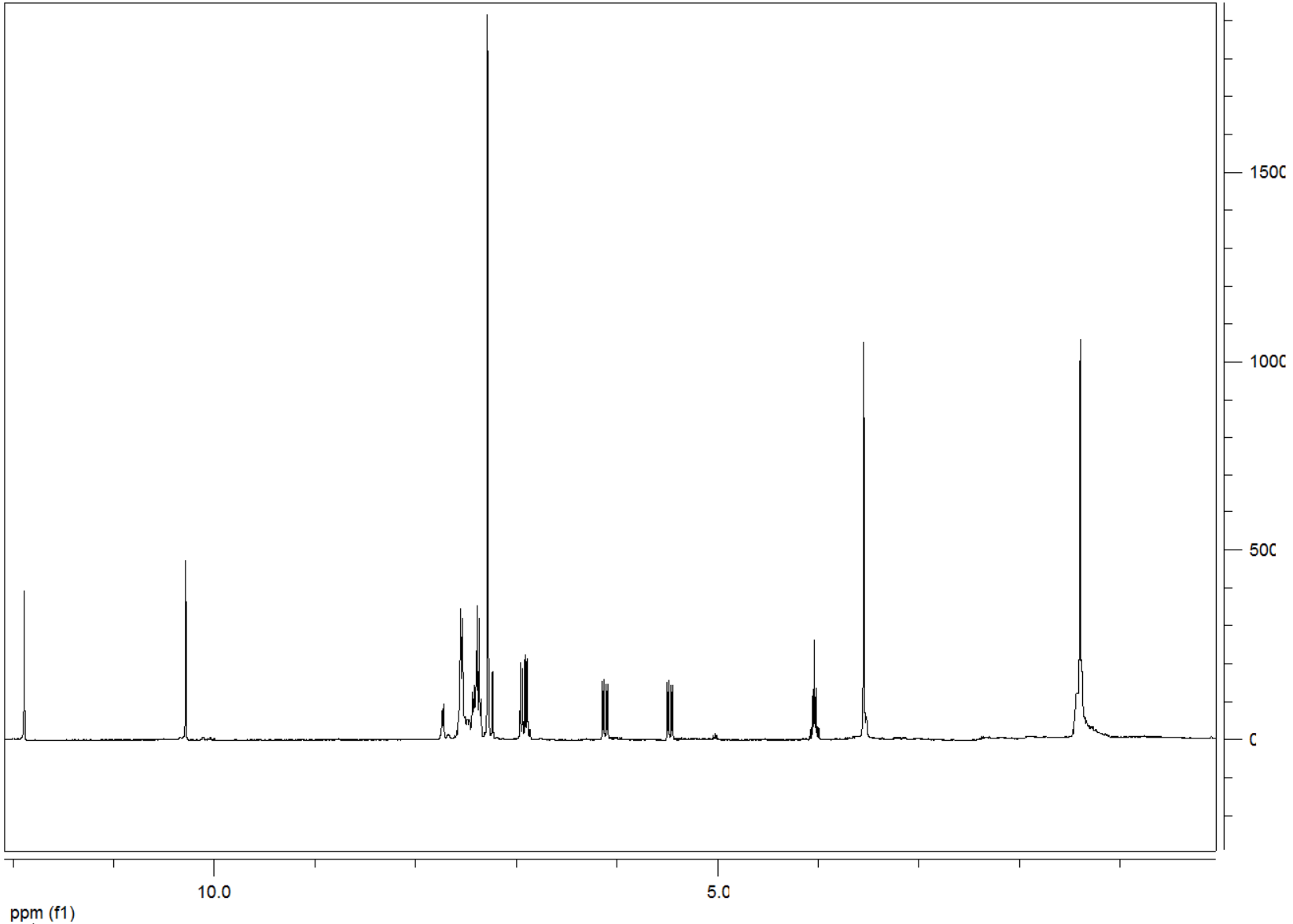


Figure 28. ^{1}H NMR spectrum of 4'-*O*-(*S*)-α-methoxy-α-trifluoromethyl-α-phenylacetate (MTPA) ester of agropyrenol (**12**) recorded in $CDCl_3$ at 400 MHz.

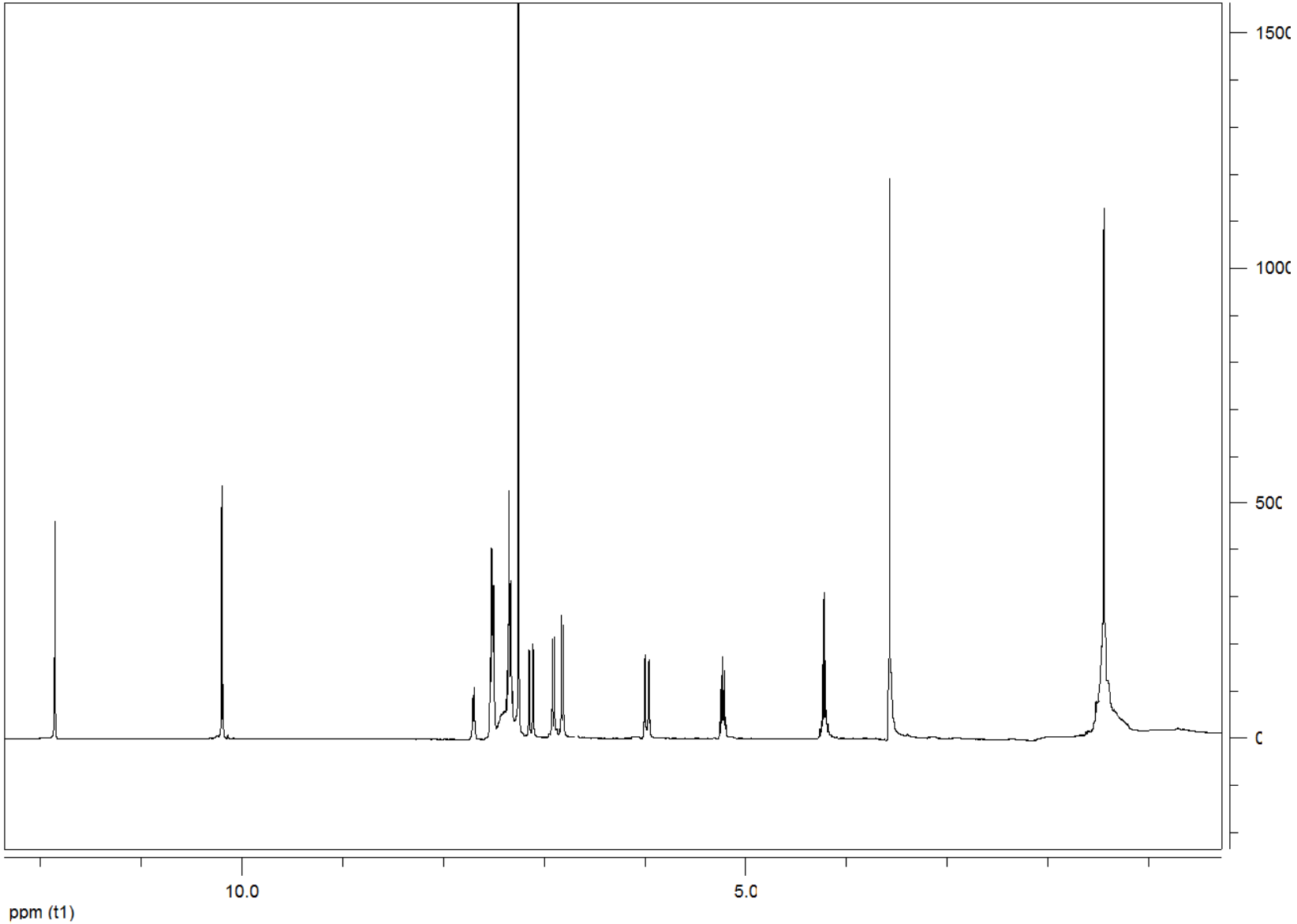
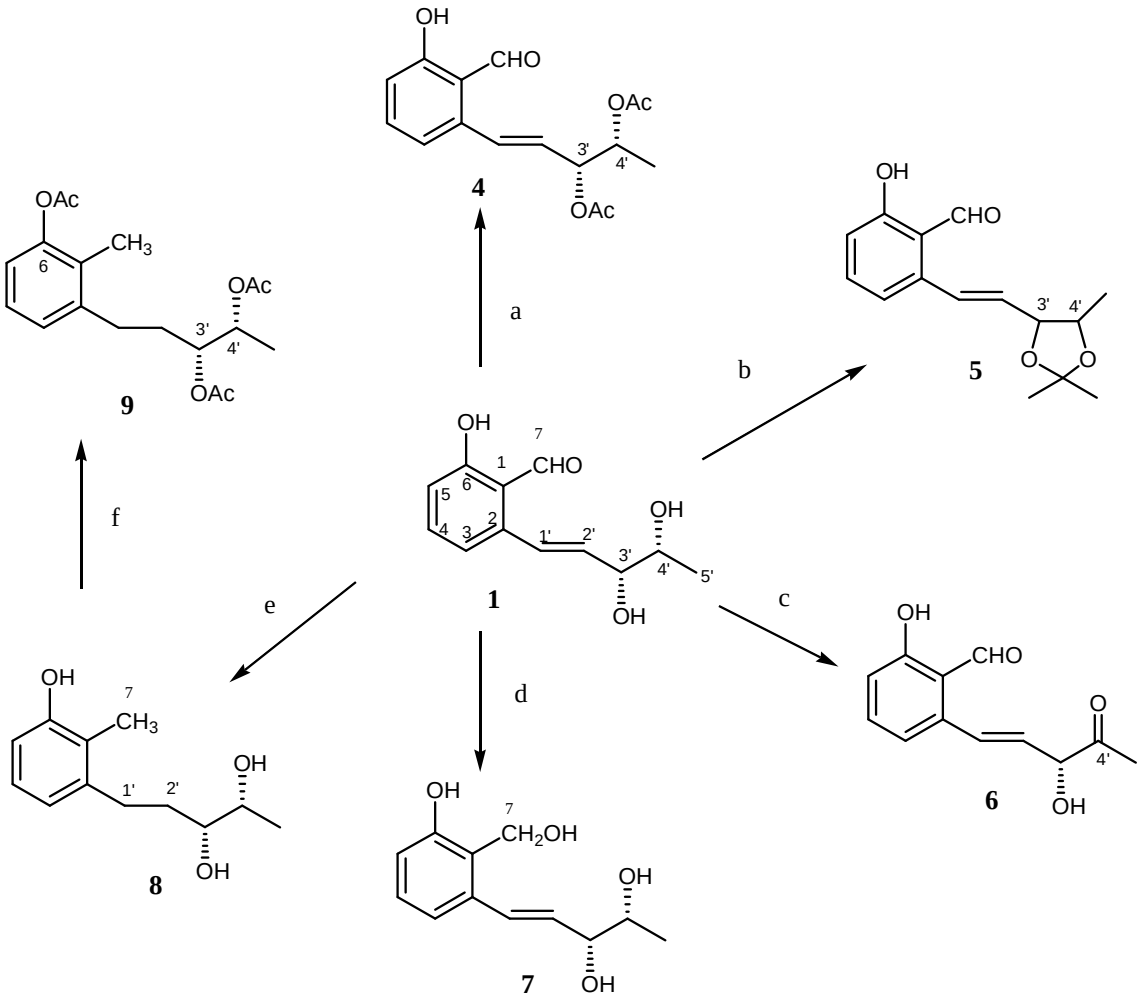


Figure 29. ^{1}H NMR spectrum of 4'-*O*-(*R*)-α-methoxy-α-trifluoromethyl-α-phenylacetate (MTPA) ester of agropyrenol (**13**) recorded in $CDCl_3$ at 400 MHz.



[a] Reagents and conditions: (a) Ac_2O, pyridine, rt, 12 h; (b) dry Me_2CO, dry $CuSO_4$, rt, 18 h; (c) MnO_2, CH_2Cl_2, 25 °C, 1.5 h; (d) $NaBH_4$, MeOH, rt, 2 h; (e) H_2, Pd/C, MeOH, rt, 1 h; (f) Ac_2O, pyridine, rt, 1.5 h

Figure 30. Hemisynthesis of agropyrenol derivatives (**4**-**9**, respectively)

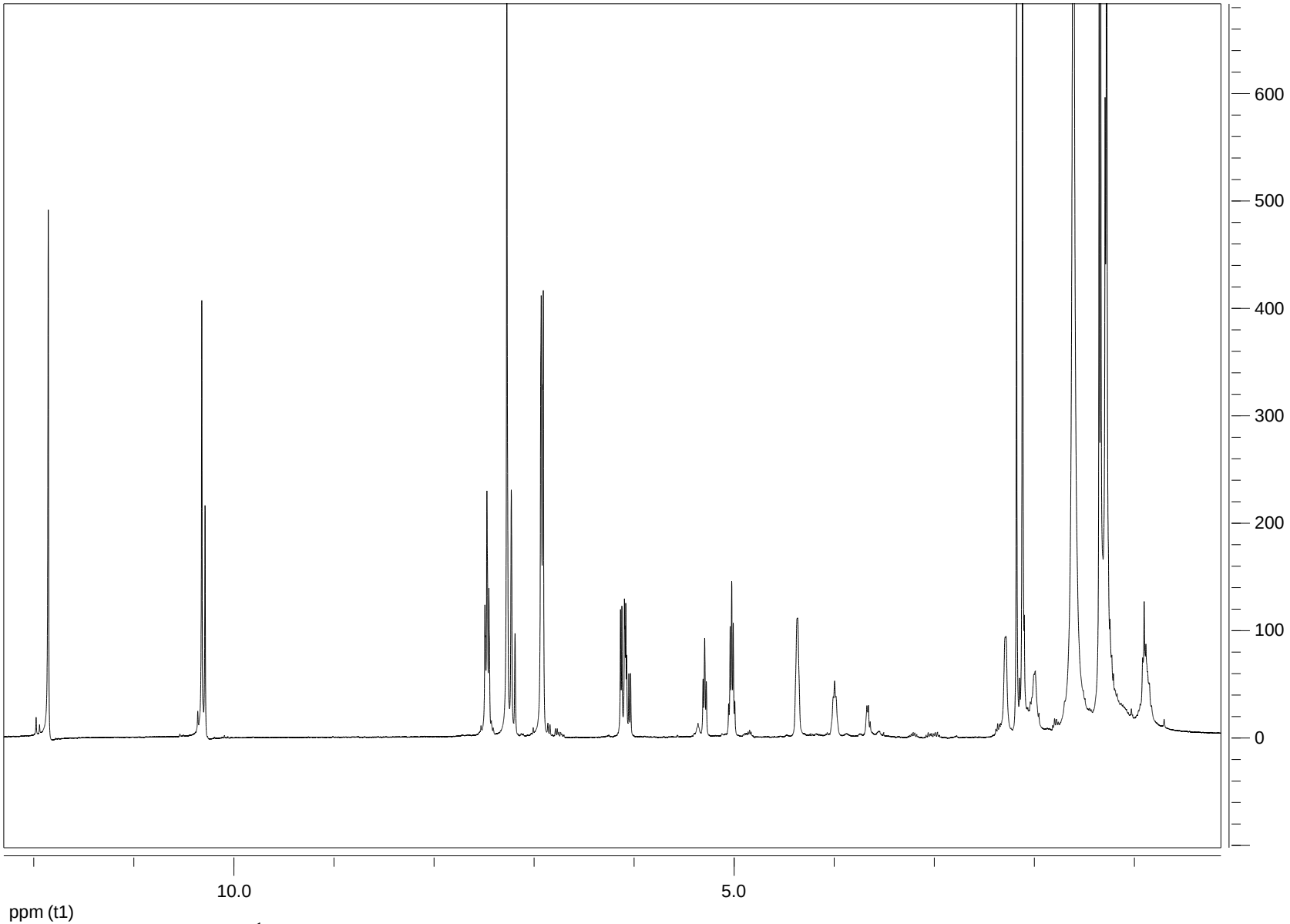


Figure 31. ^{1}H NMR spectrum of 3',4'-*O*,*O*'-diacetyl agropyrenol (**4**) recorded in $CDCl_3$ at 400 MHz.

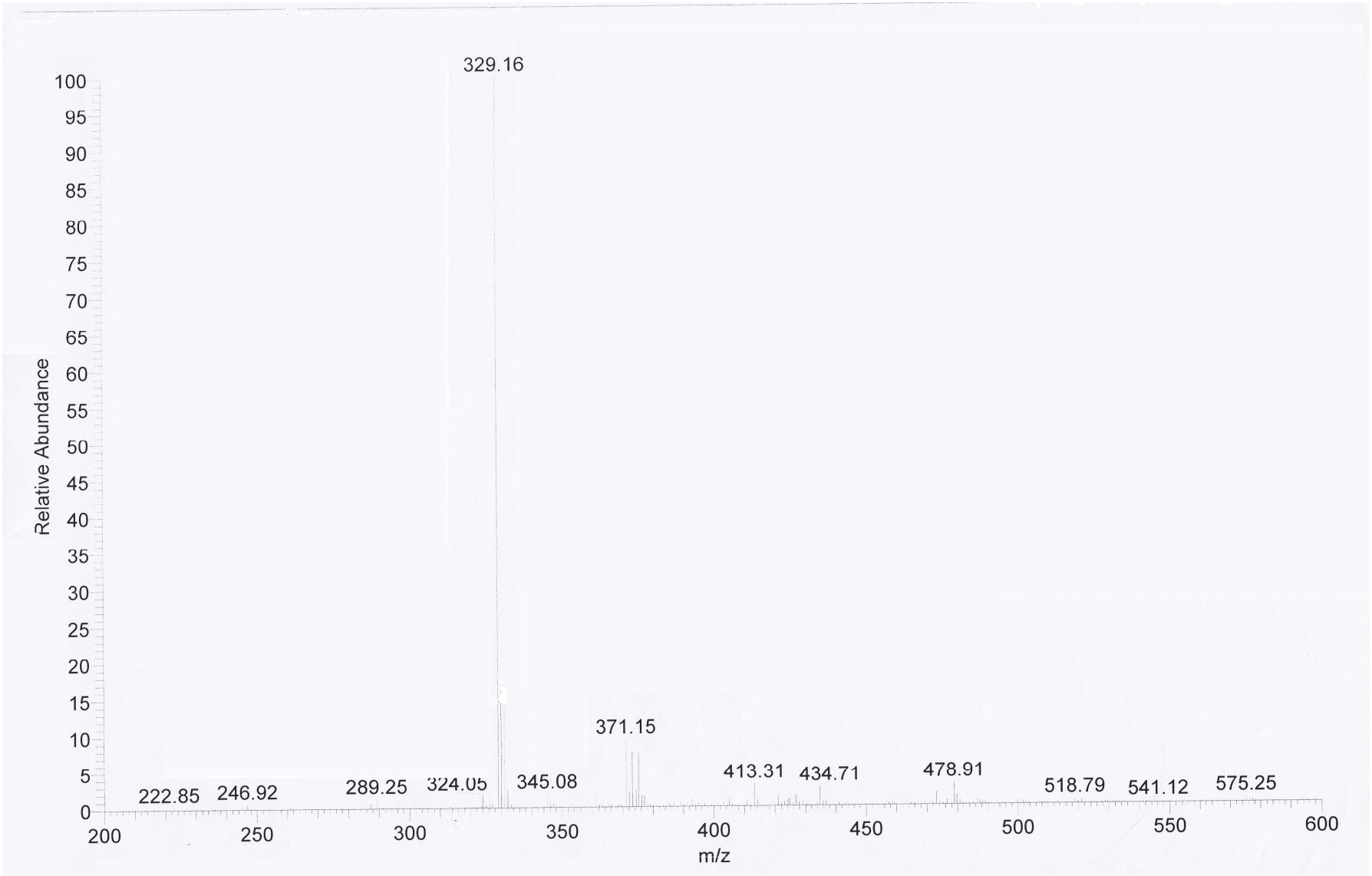


Figure 32. HR-ESIMS spectrum of 3',4'-*O,O'*-diacetyl agropyrenol (**4**) recorded in positive modality.

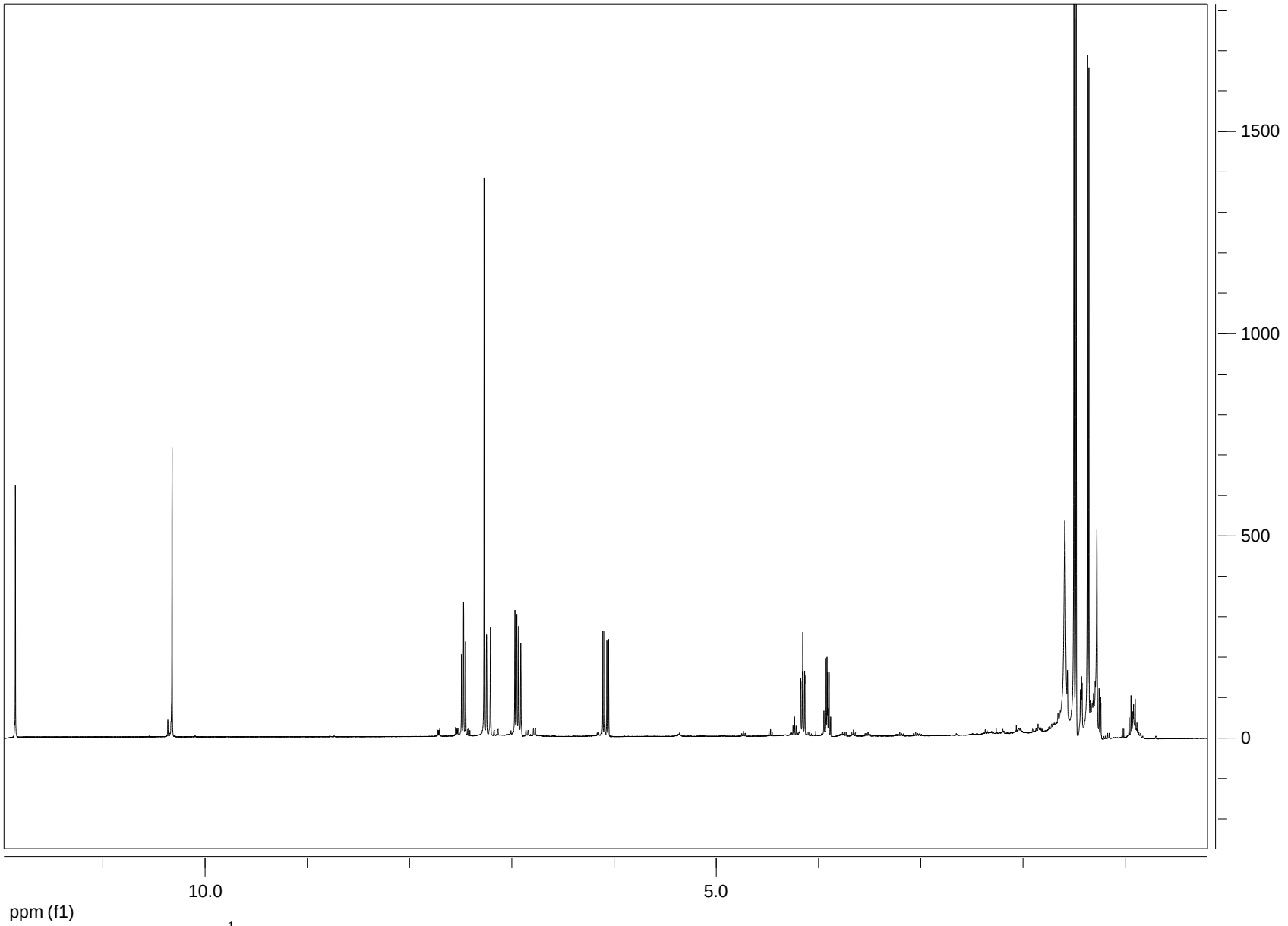


Figure 33. ^{1}H NMR spectrum of 3',4'-*O*,*O*'-isopropylideneagropyrenol (**5**) recorded in $CDCl_3$ at 400 MHz.

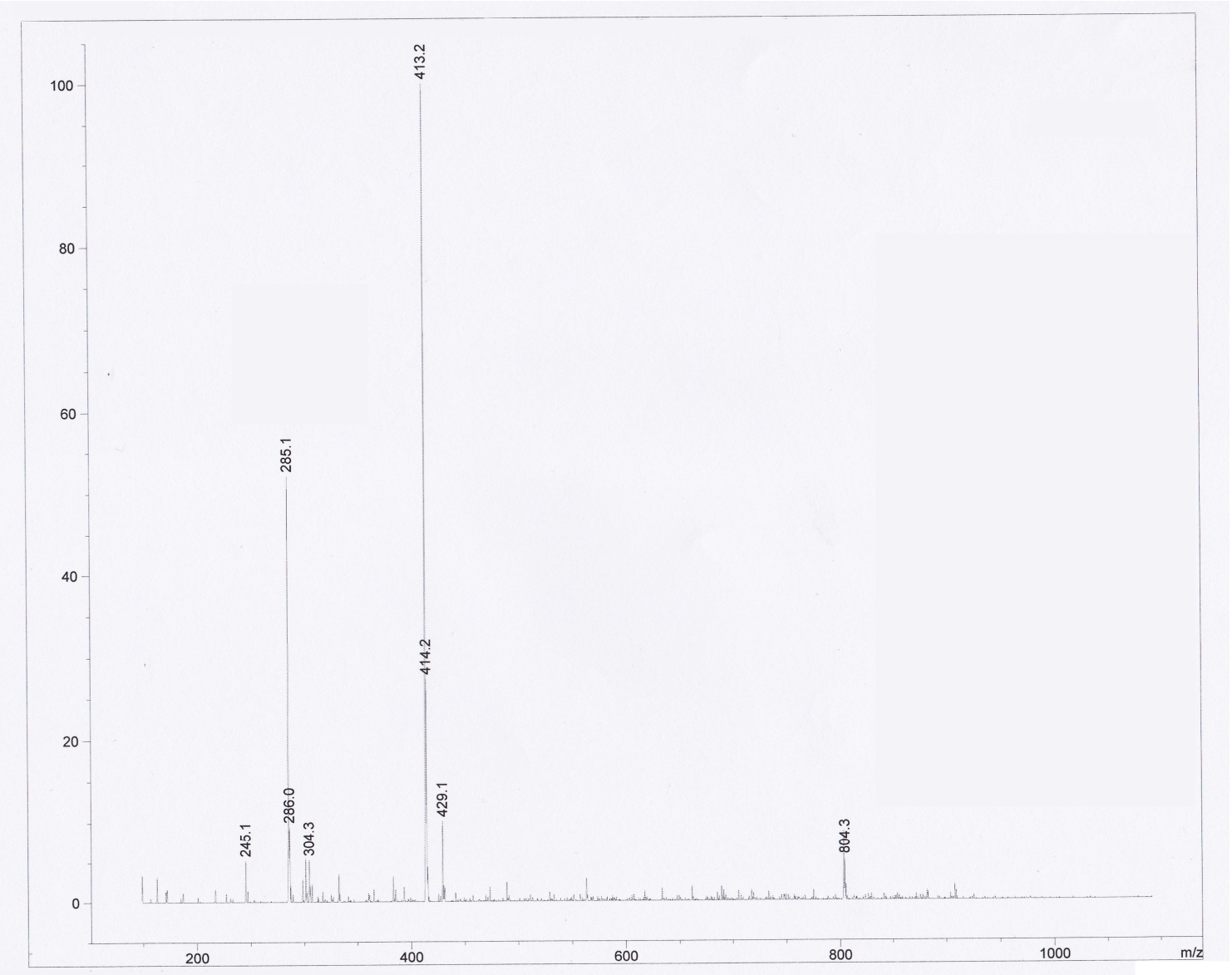


Figure 34. HR-ESIMS spectrum of 3',4'-*O*,*O'*-isopropylideneagropyrenol (**5**) recorded in positive modality.

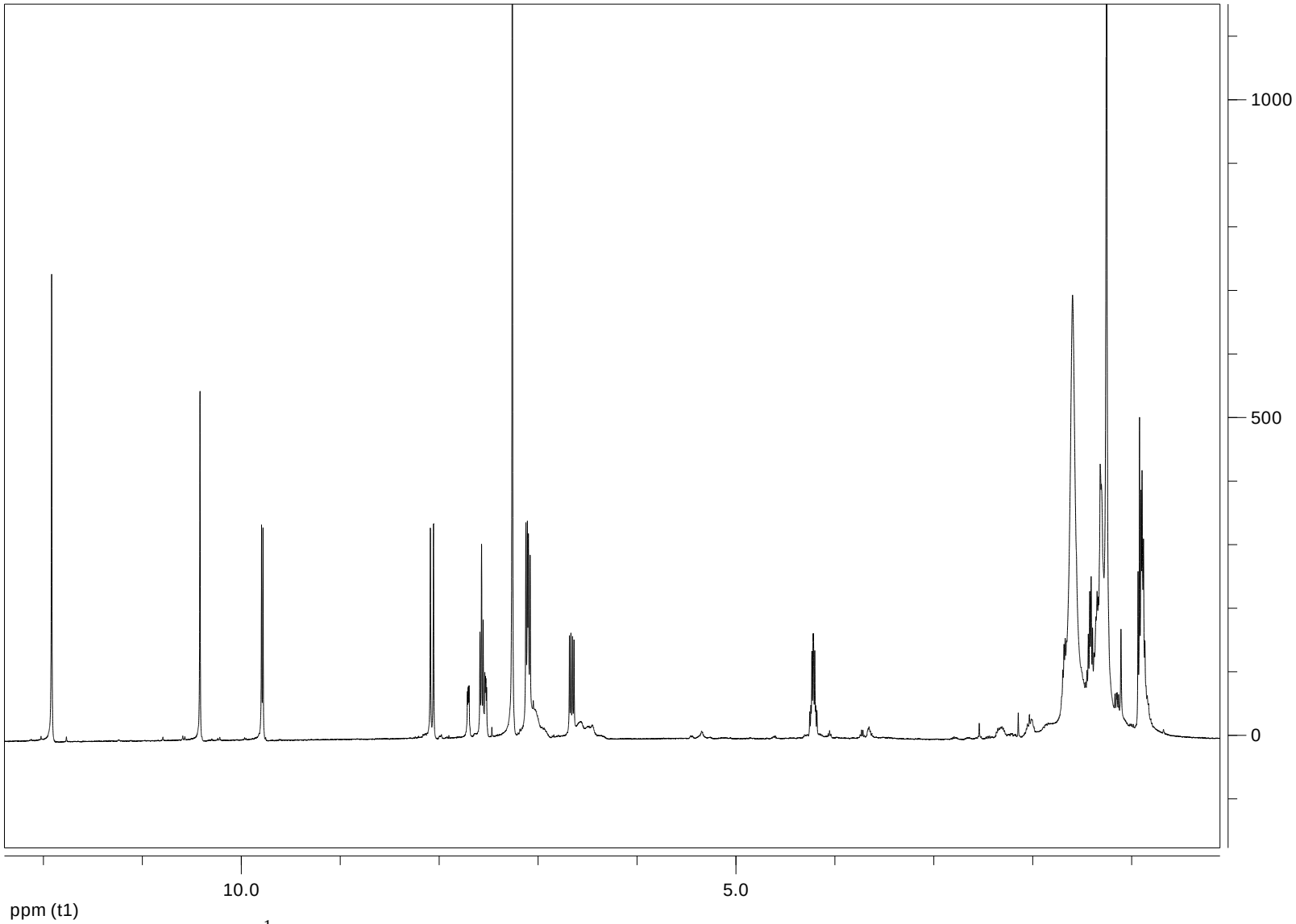


Figure 35. ^{1}H NMR spectrum of 4'-*O*-didehydroagropyrenol (**6**) recorded in $CDCl_3$ at 400 MHz.

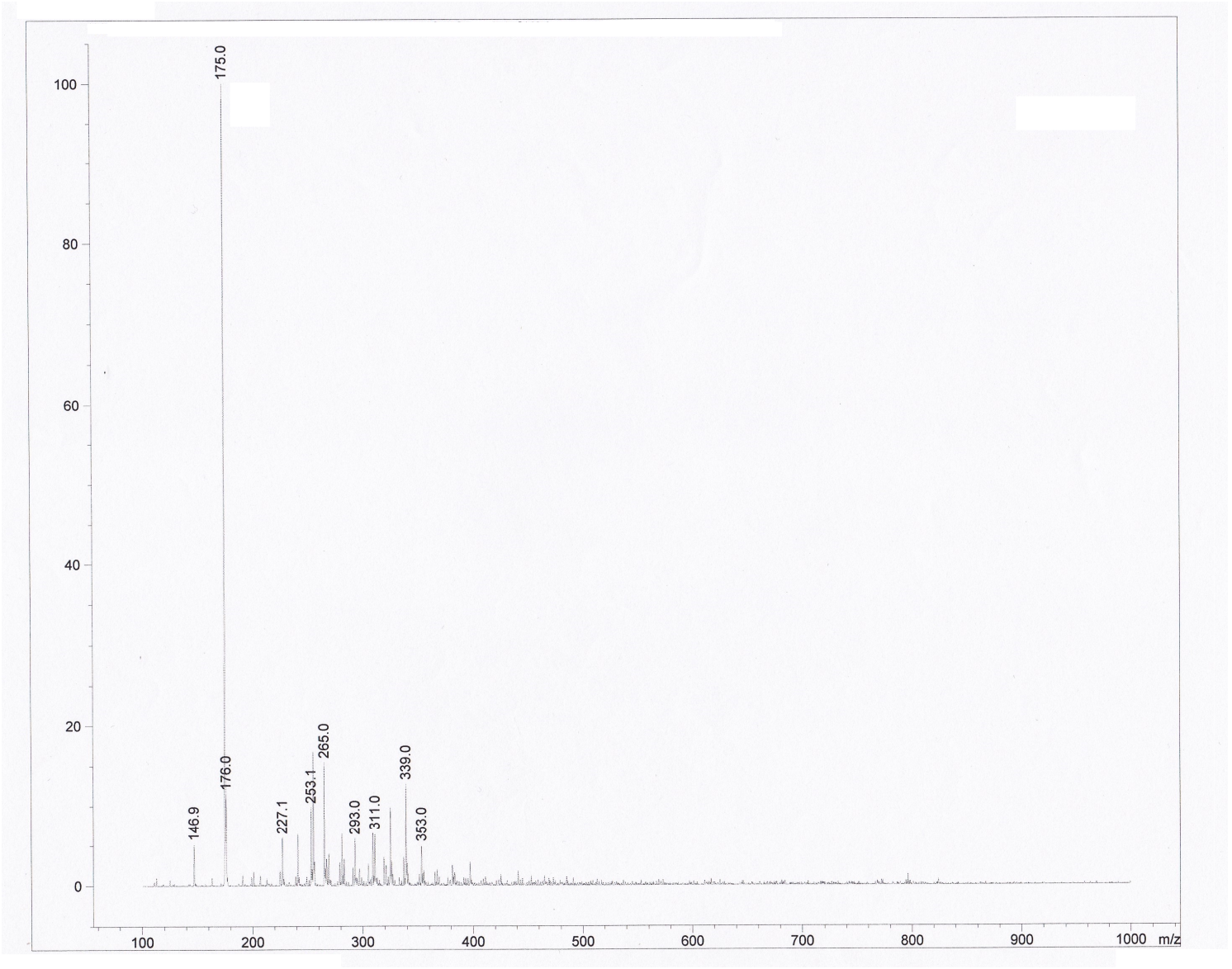


Figure 36. HR-ESIMS spectrum of 4'-*O*-didehydroagropyrenol (**6**) recorded in negative modality.

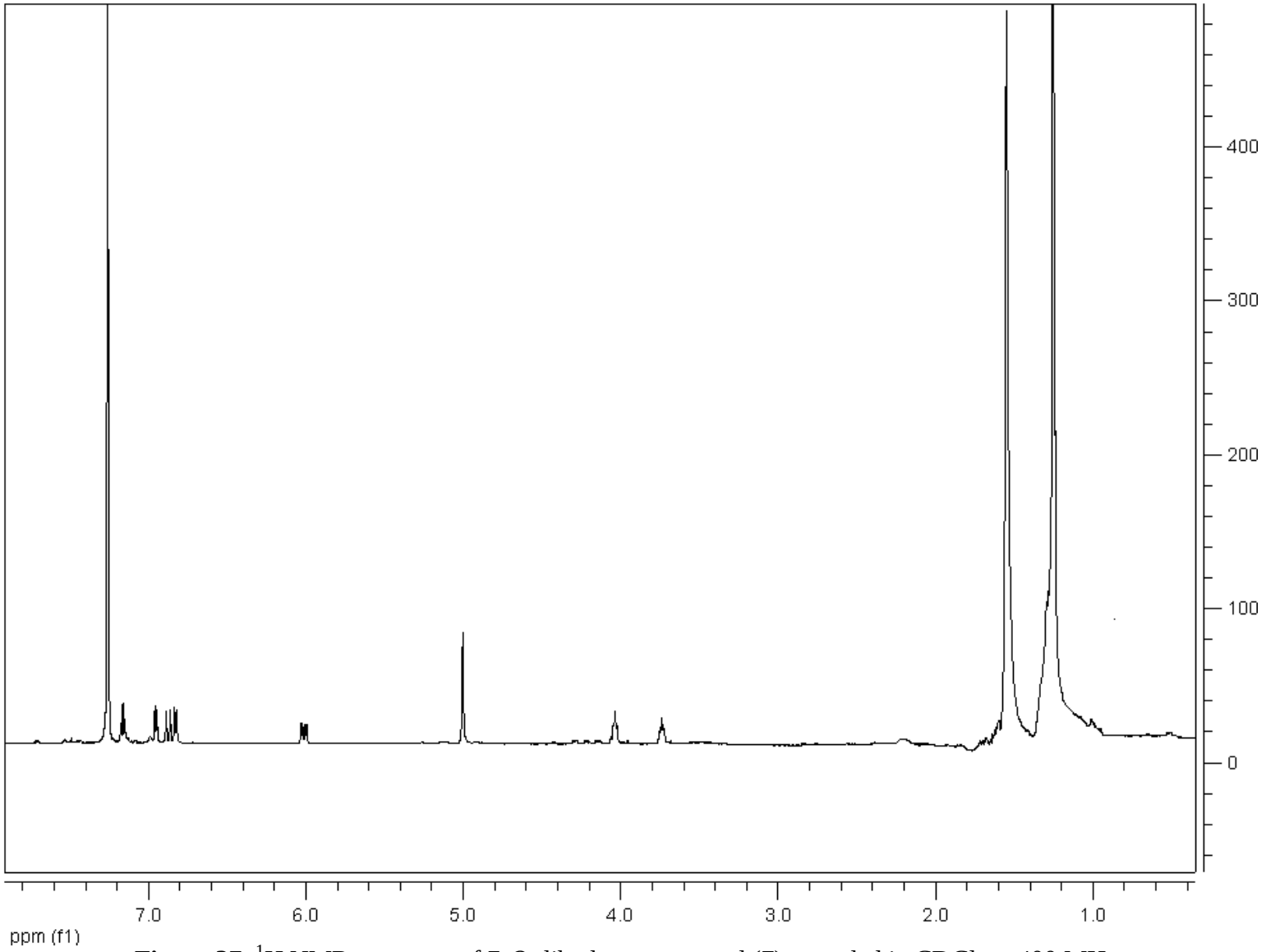


Figure 37. ^{1}H NMR spectrum of 7-*O*-dihydroagropyrenol (**7**) recorded in $CDCl_3$ at 400 MHz.

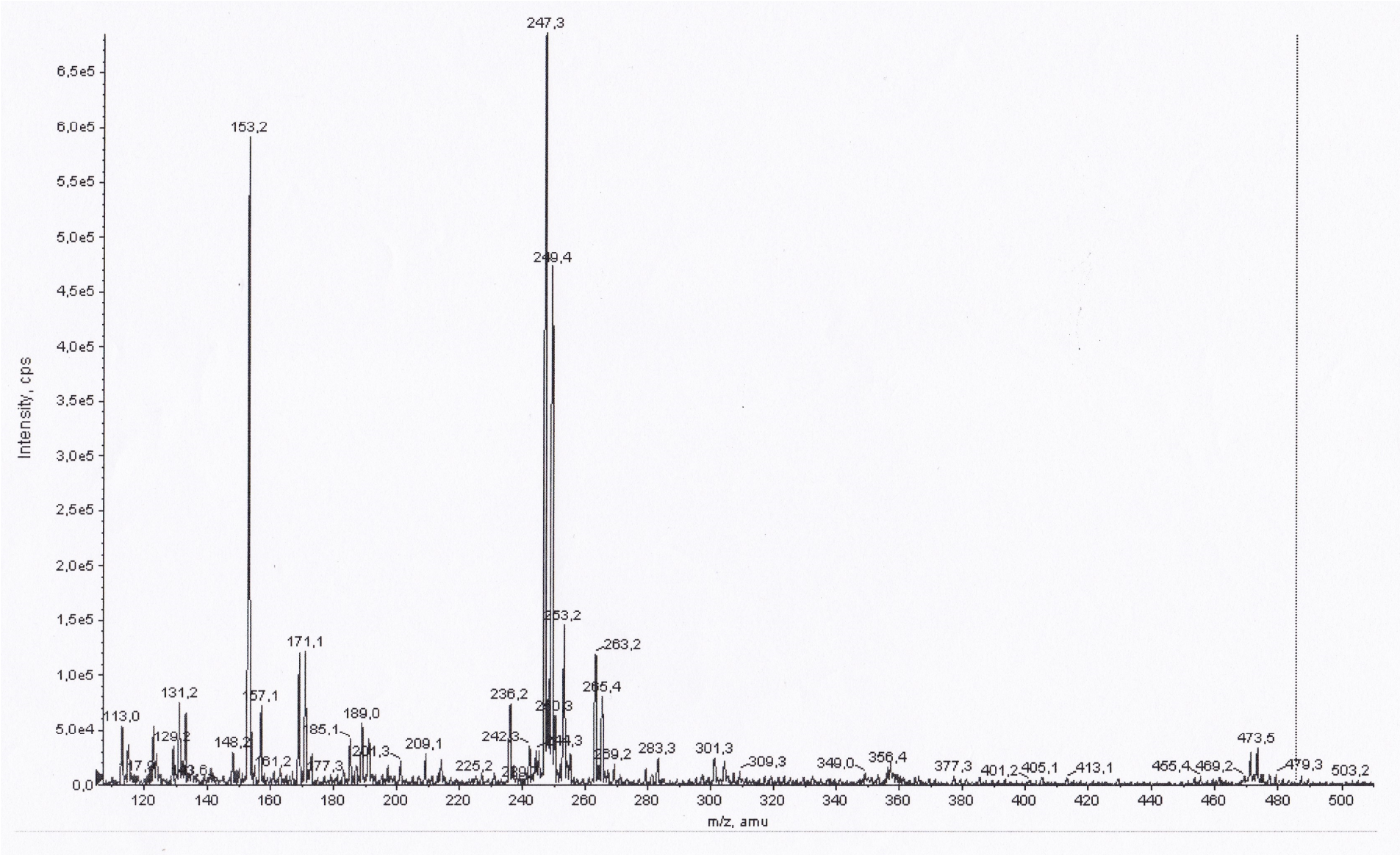


Figure 38. HR-ESIMS spectrum of 7-*O*-dihydroagropyrenol (**7**) recorded in positive modality.

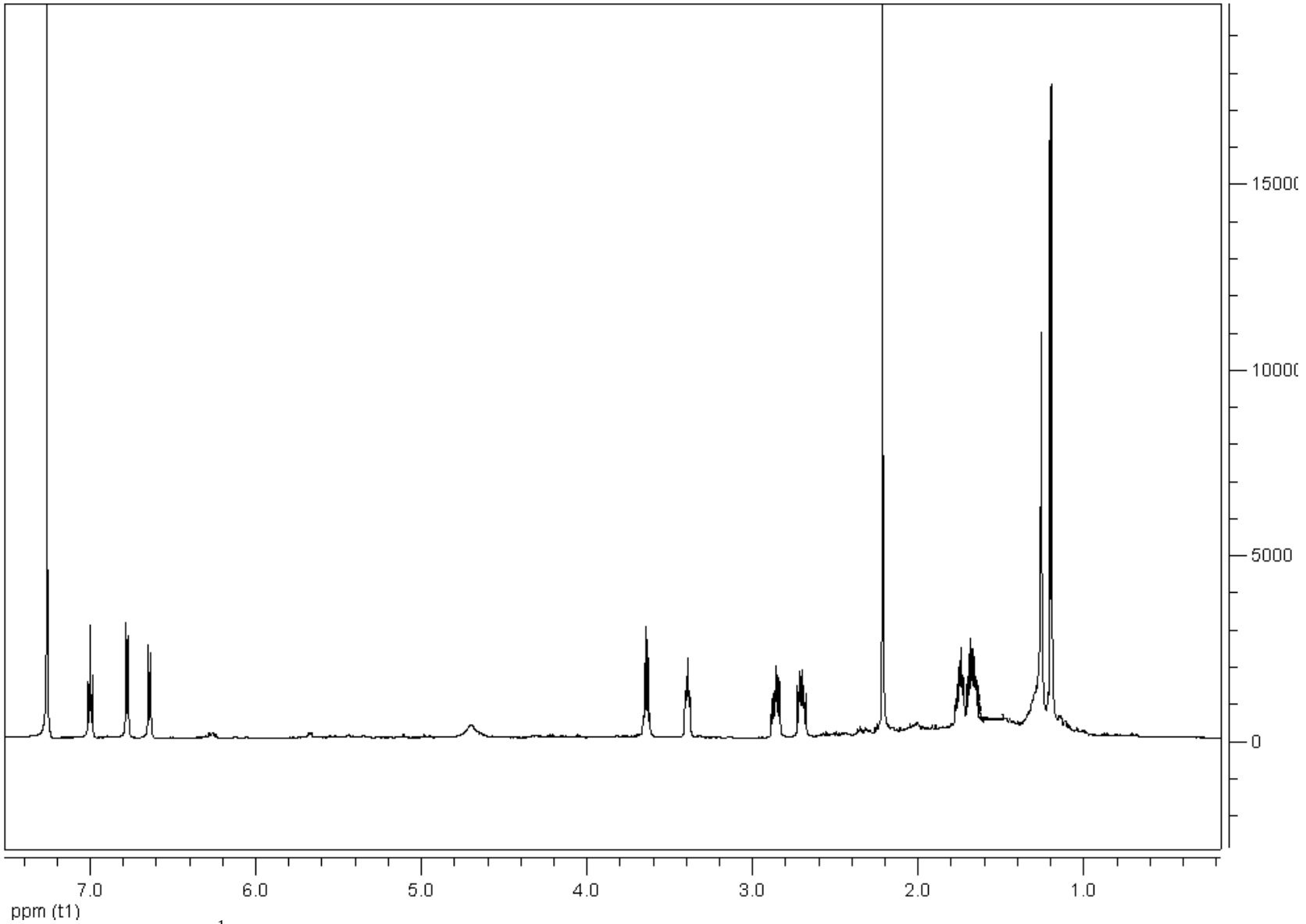


Figure 39. ^{1}H NMR spectrum of 7,7,1'2'-tetrahydro-7-deoxyagropyrenol (**8**) recorded in $CDCl_3$ at 400 MHz.

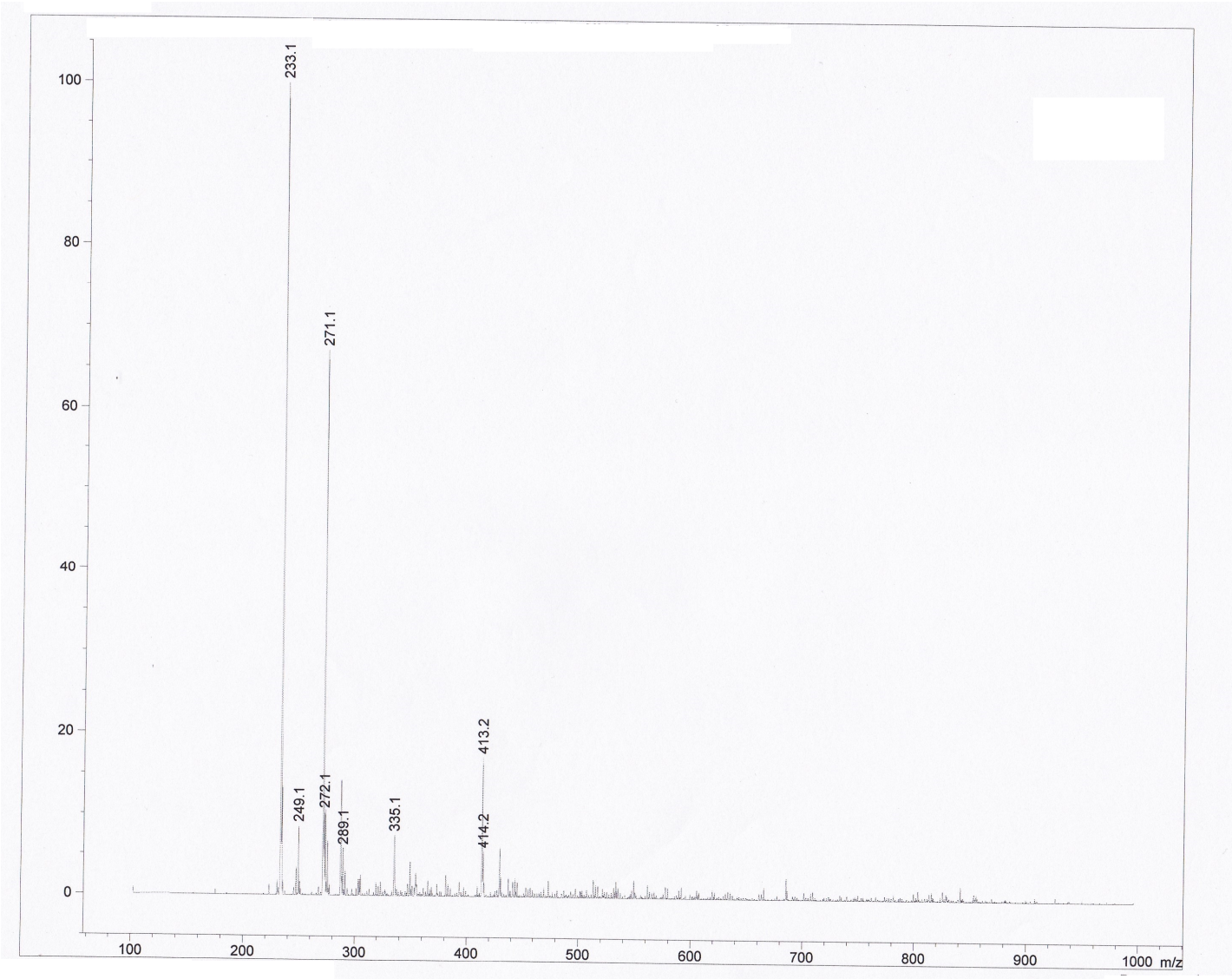


Figure 40. ^{1}H NMR spectrum of 7,7,1'2'-tetrahydro-7-deoxyagropyrenol (**8**) recorded in positive modality.

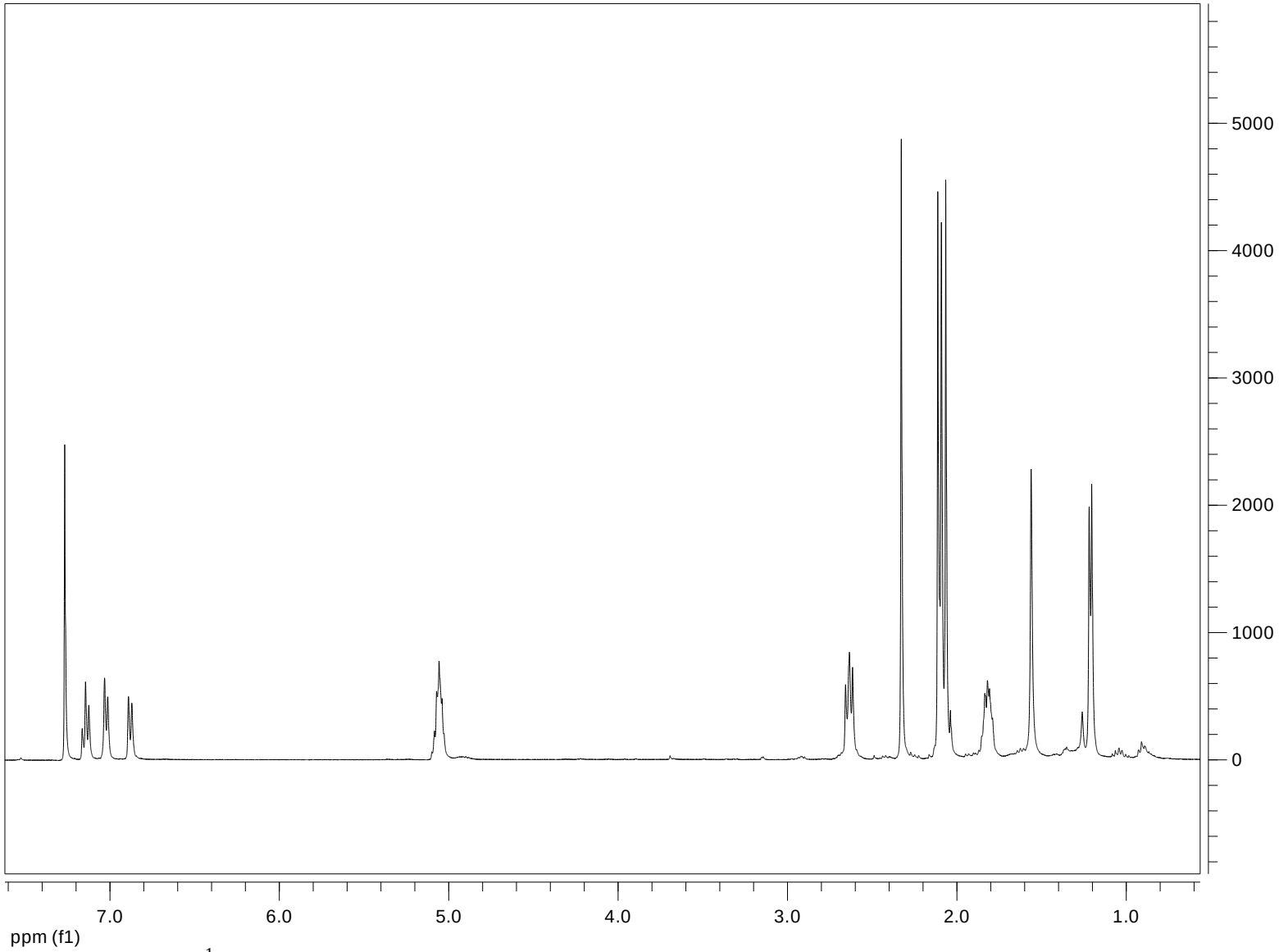


Figure 41. ^{1}H NMR spectrum of 6,3',4'-*O,O',O"*-triacetyl derivative (**9**) recorded in $CDCl_3$ at 400 MHz.

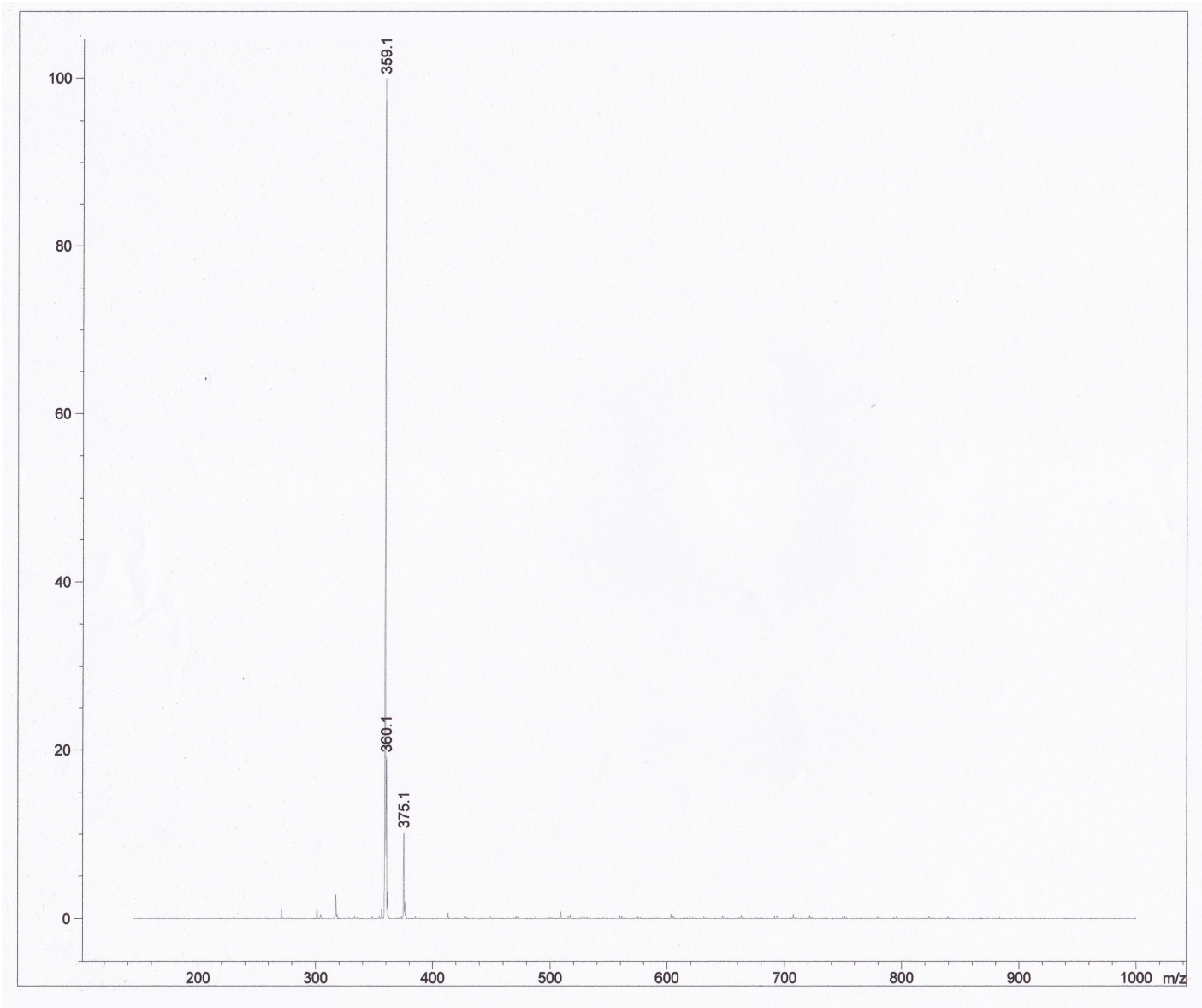


Figure 42. [1]H NMR spectrum of 6,3',4'-*O*,*O'*,*O"*-triacetyl derivative (**9**) recorded in positive modality.

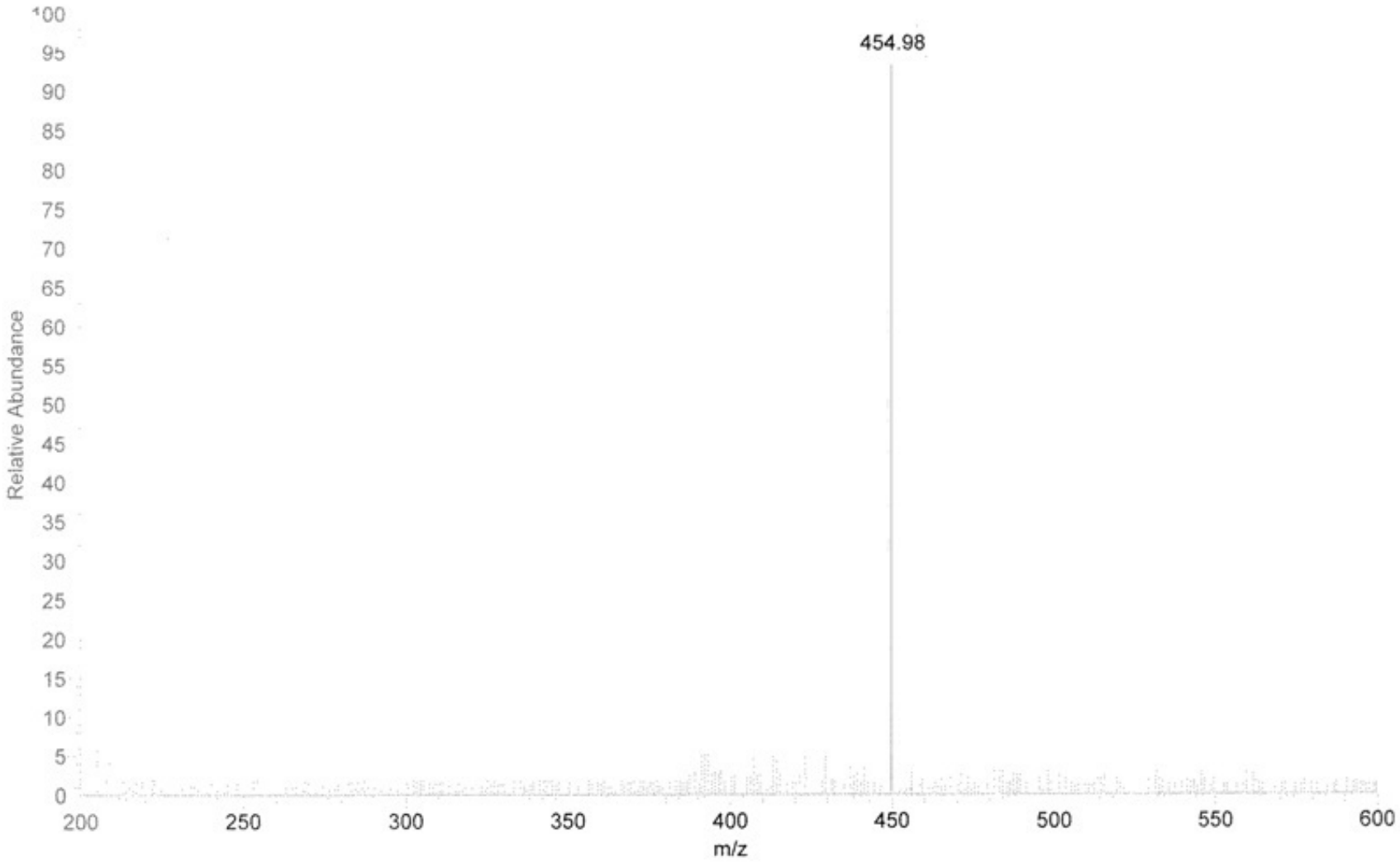


Figure 43. ESI MS spectrum of agropyrenale (**2**) recorded in positive modality.

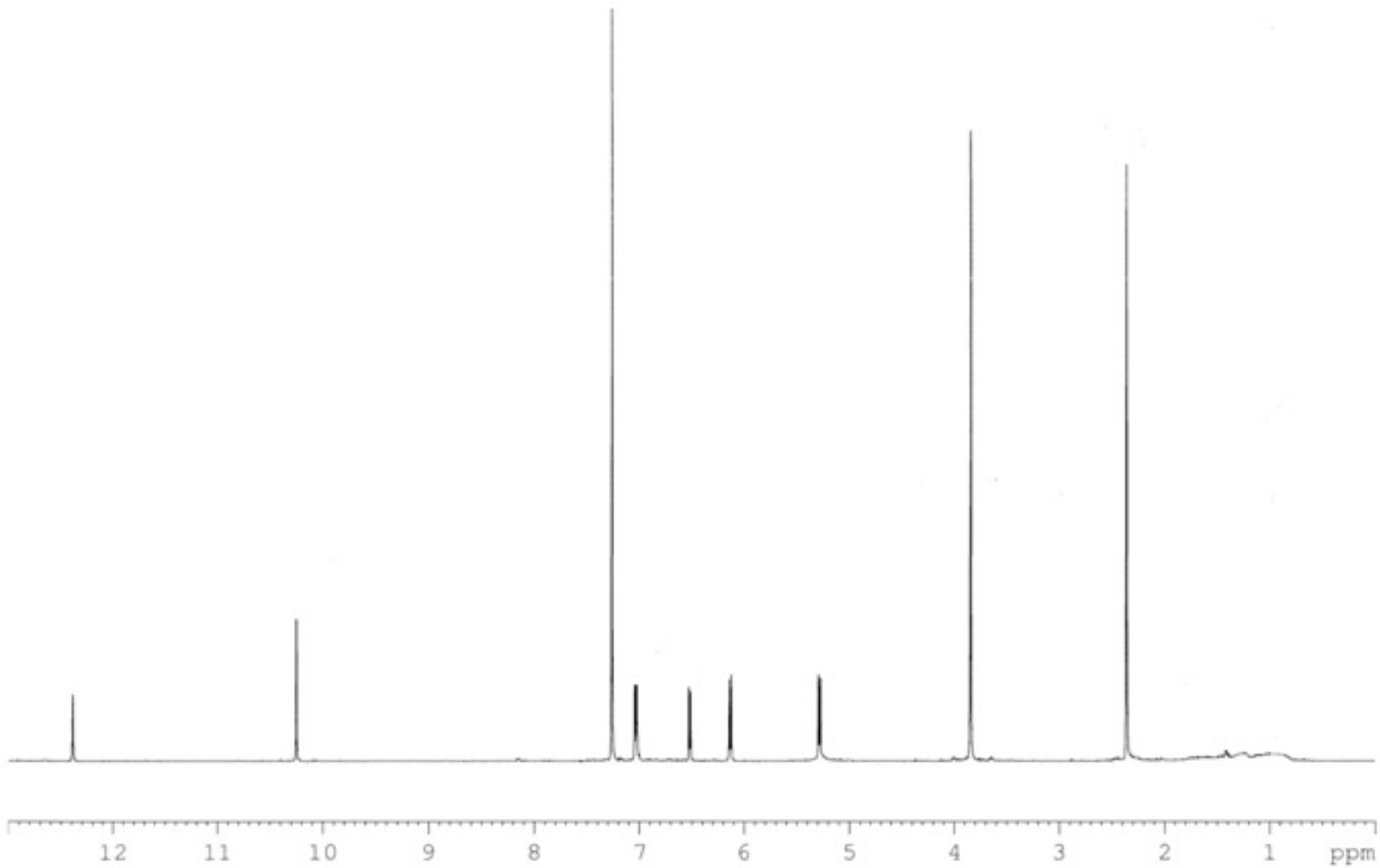


Figure 44. ^{1}H NMR spectrum of agropyrenale (**2**) recorded in $CDCl_3$ at 400 MHz.

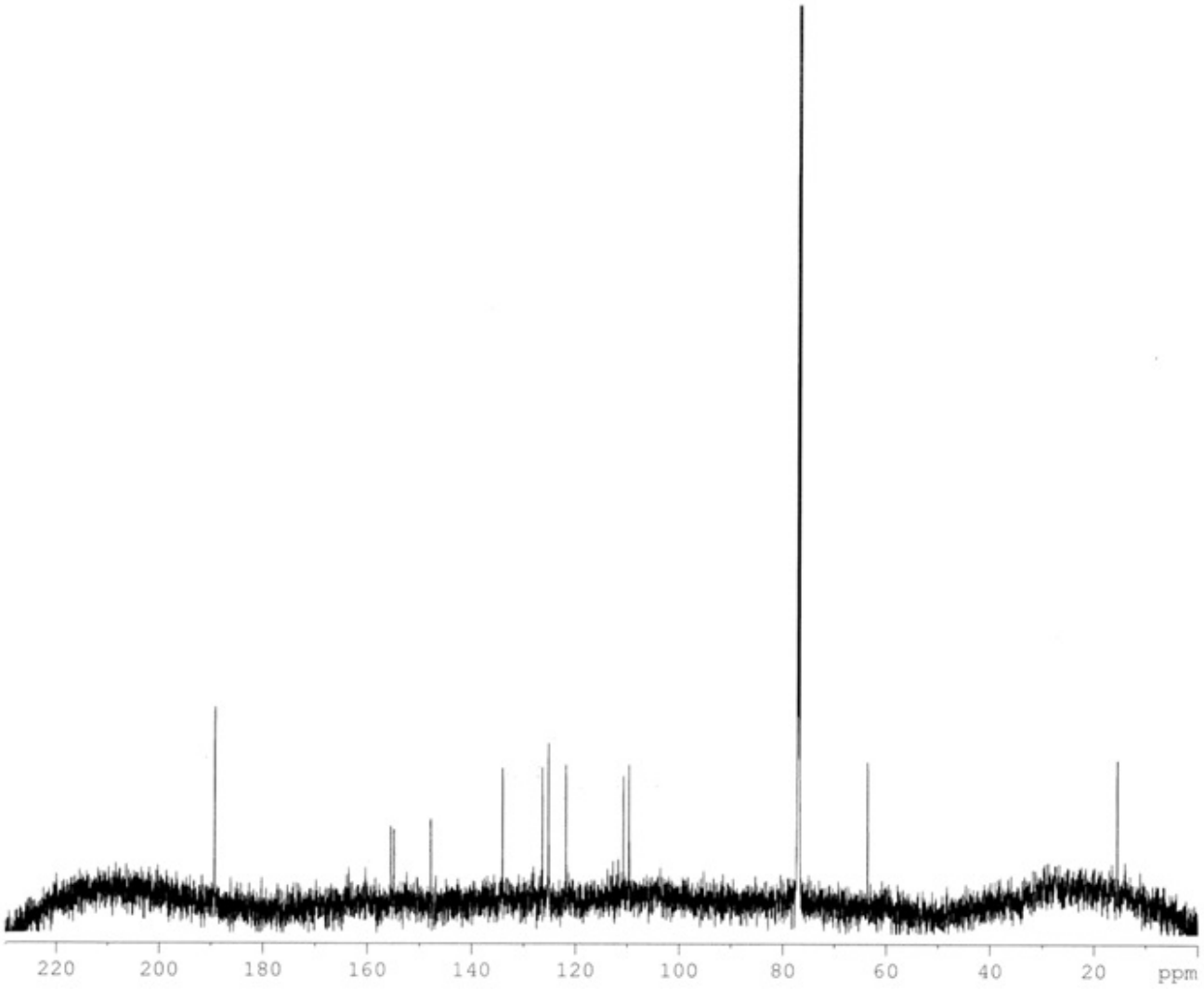


Figure 45. ^{13}C NMR spectrum of agropyrenale (**2**) recorded in $CDCl_3$ at 400 MHz.

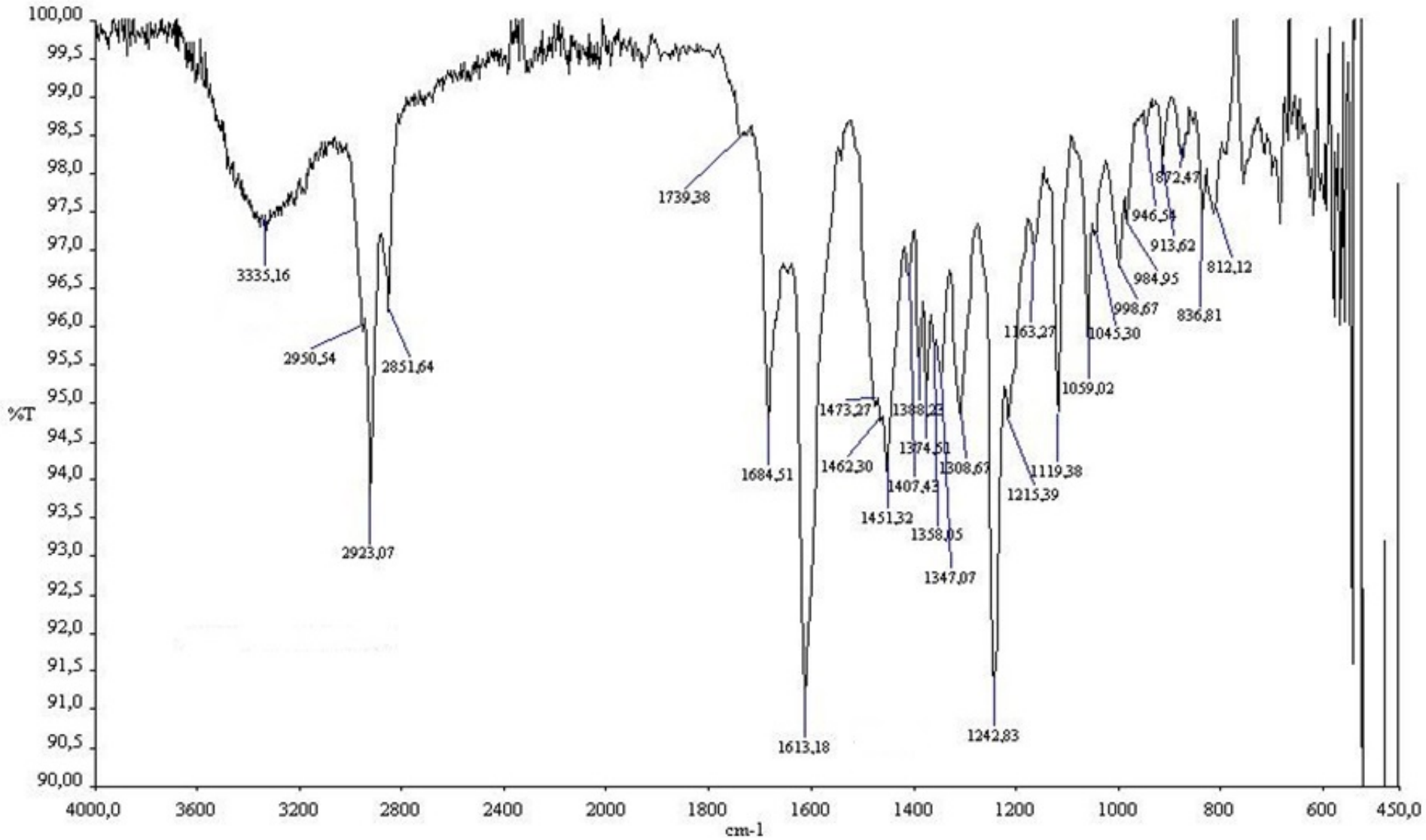


Figure 46. IR spectrum of agropyrenale (**2**) recorded as glass liquid.

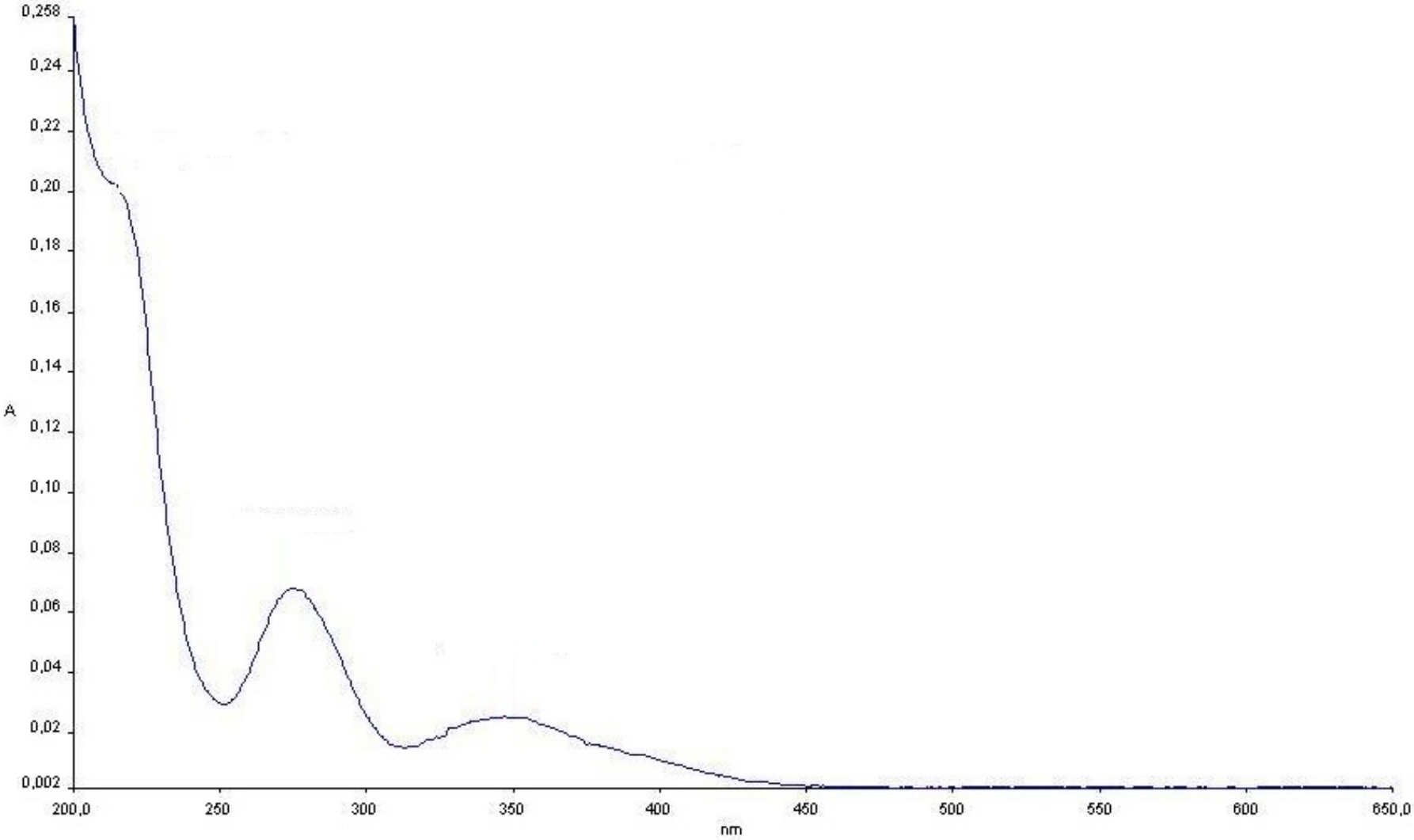


Figure 47. UV spectrum of agropyrenale (**2**) recorded in MeCN solution.

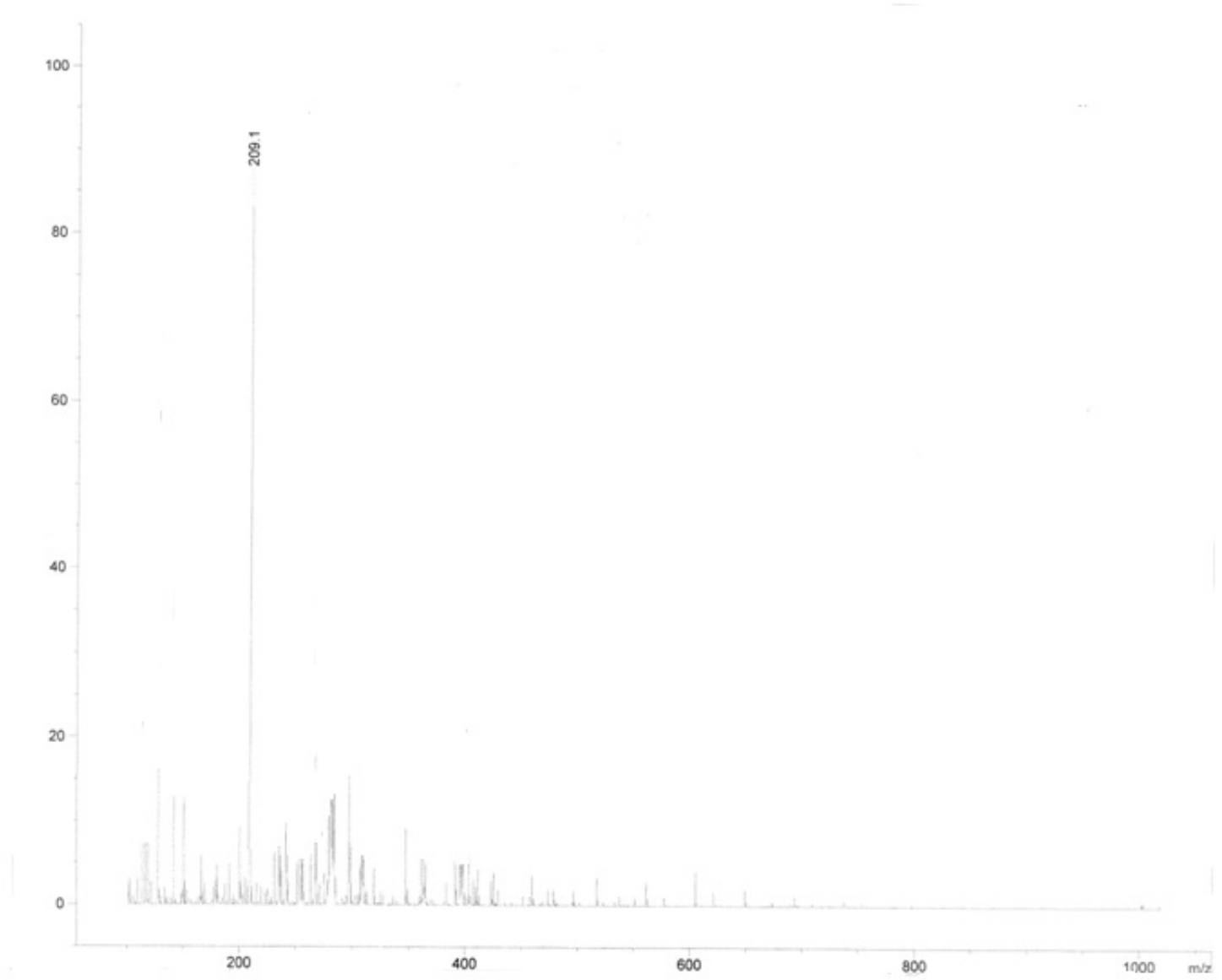


Figure 48. HR-ESIMS spectrum of agropyrenone (**3**) recorded in positive modality.

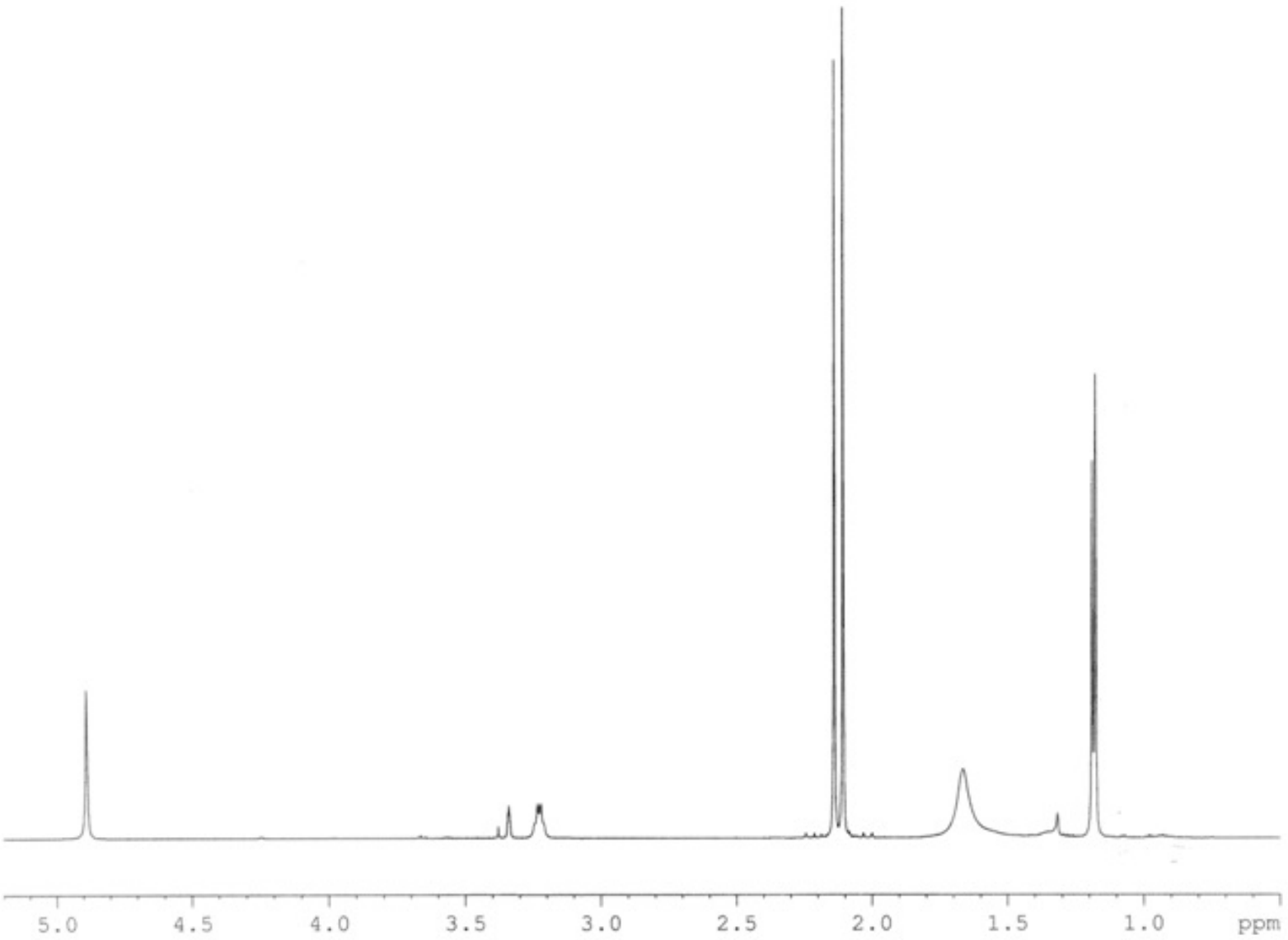


Figure 49. ^{1}H NMR spectrum of agropyrenone (**3**) recorded in CD_3OD at 400 MHz.

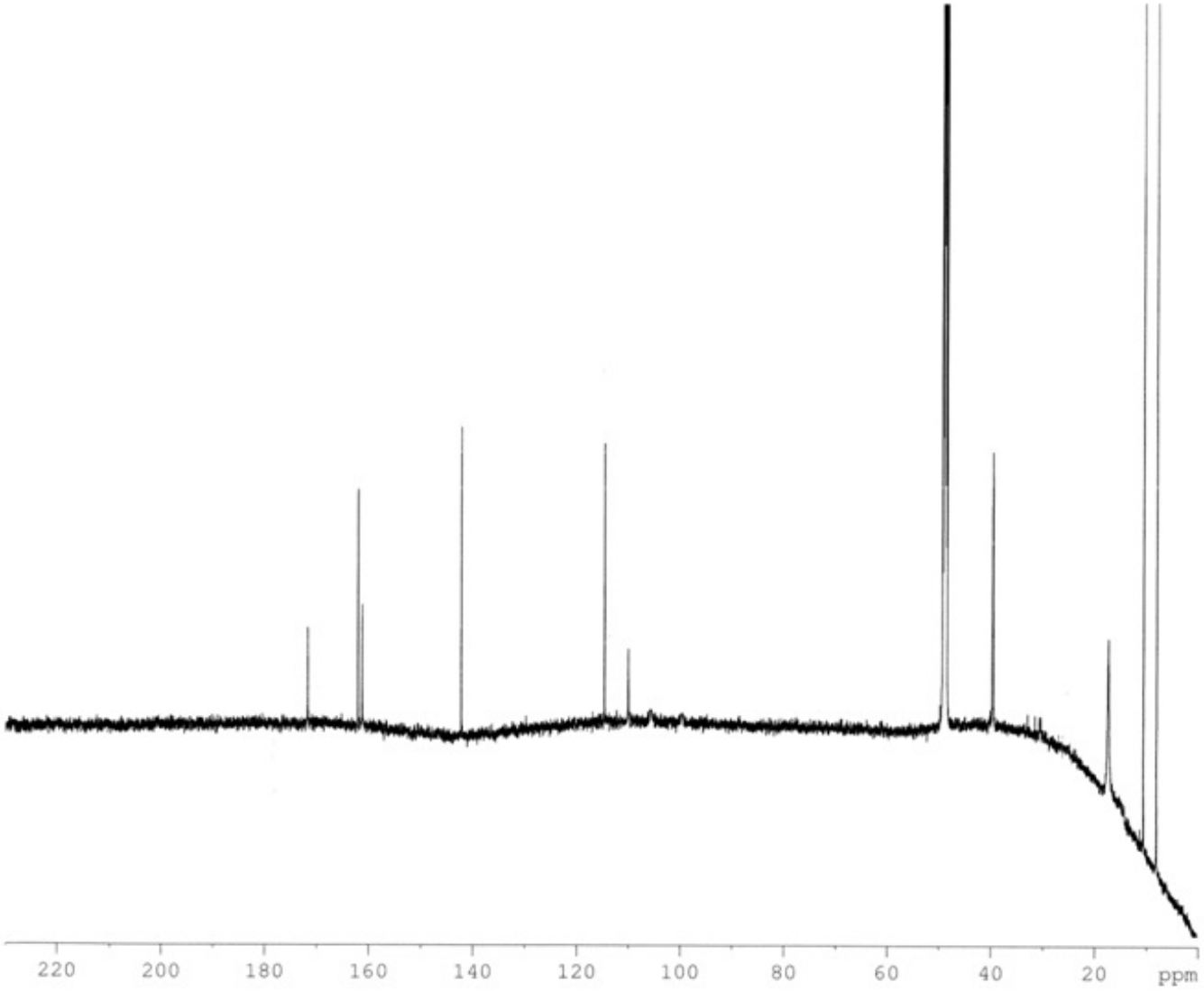


Figure 50. ^{13}C NMR spectrum of agropyrenone (**3**) recorded in CD_3OD at 400 MHz.

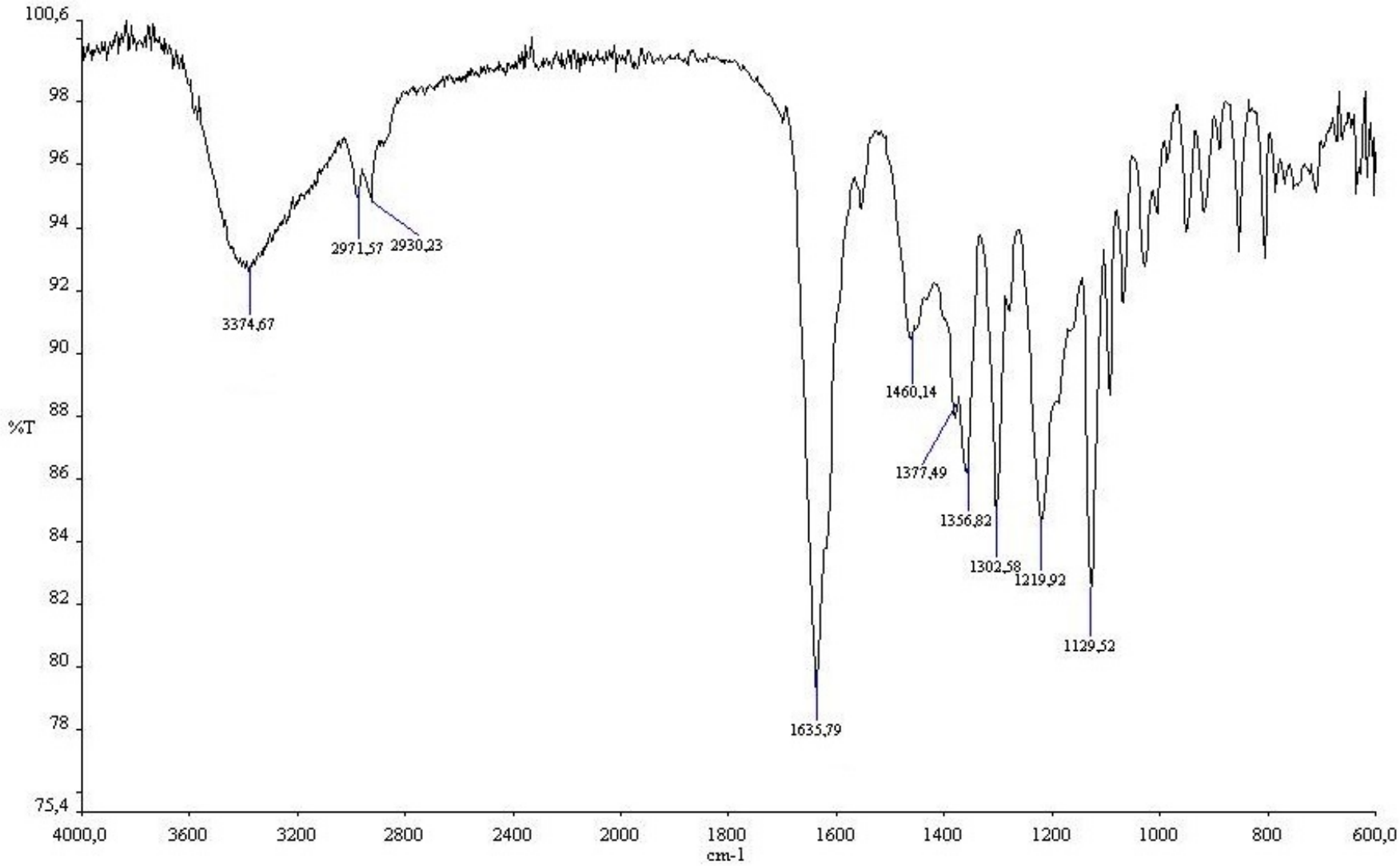


Figure 51. IR spectrum of agropyrenone (**3**) recorded as glass liquid.

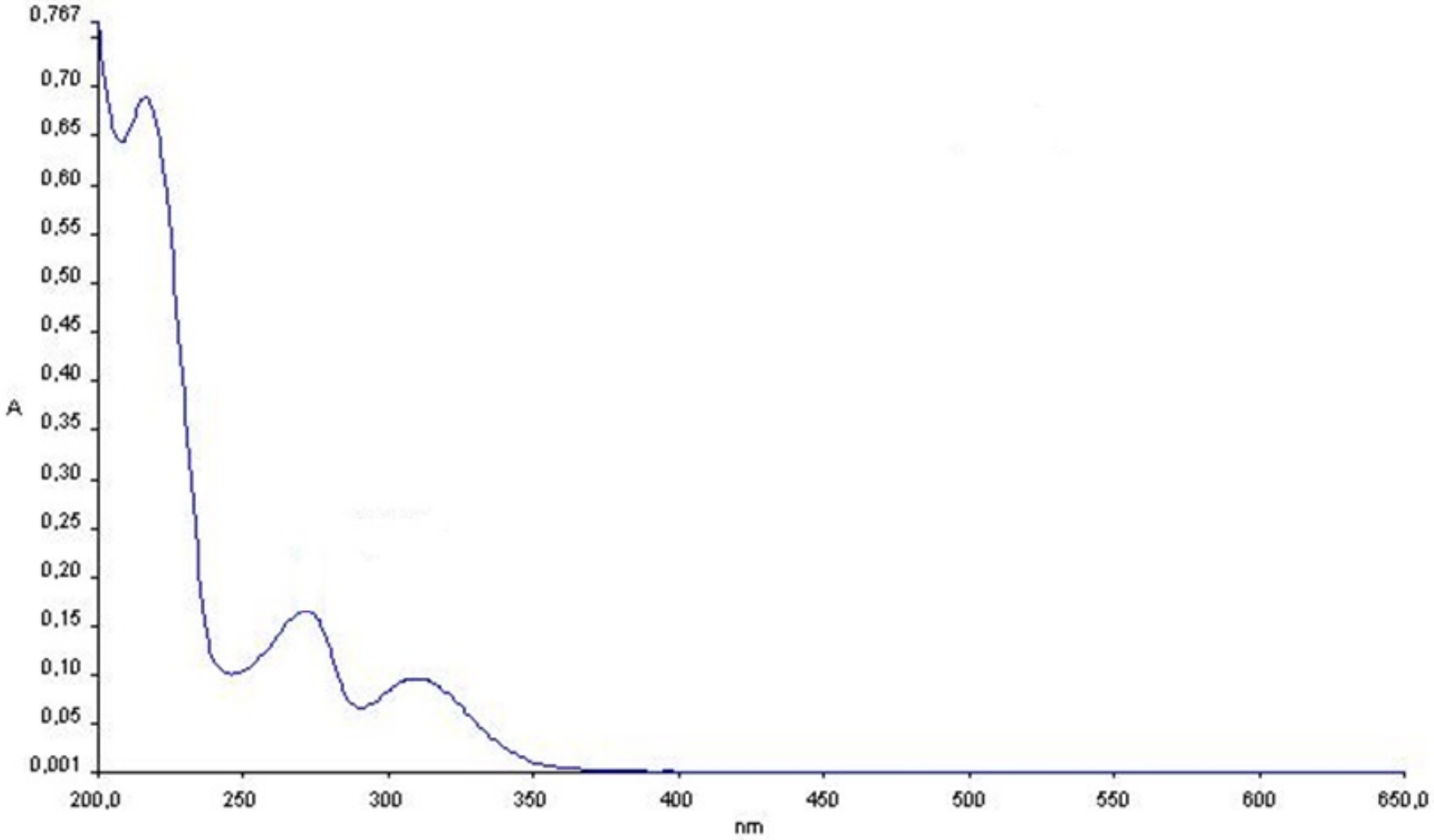


Figure 52. UV spectrum of agropyrenone (**3**) recorded in MeCN solution.

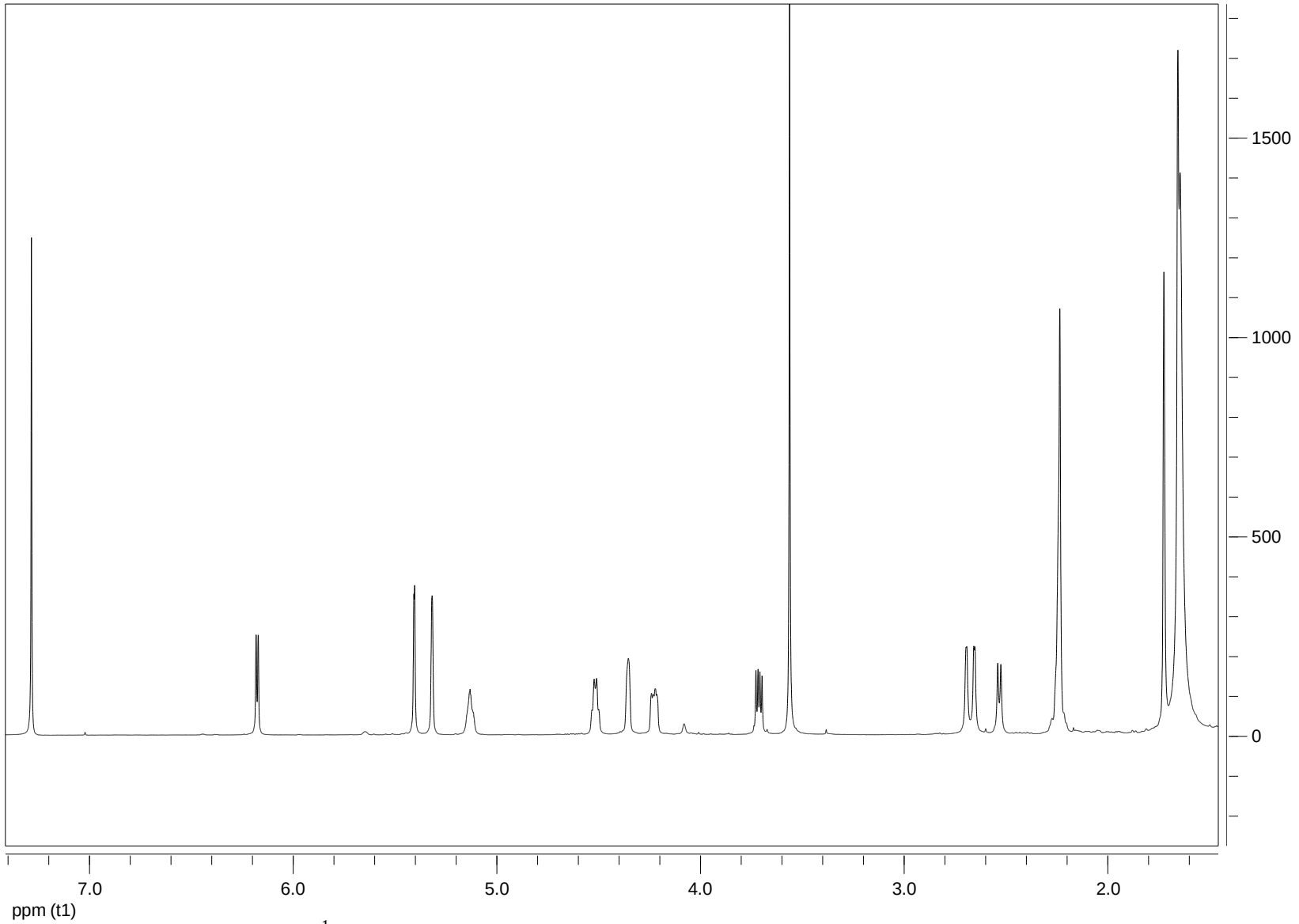


Figure 53. ^{1}H NMR spectrum of phomentrioloxin (**14**) recorded in $CDCl_3$ at 400 MHz.

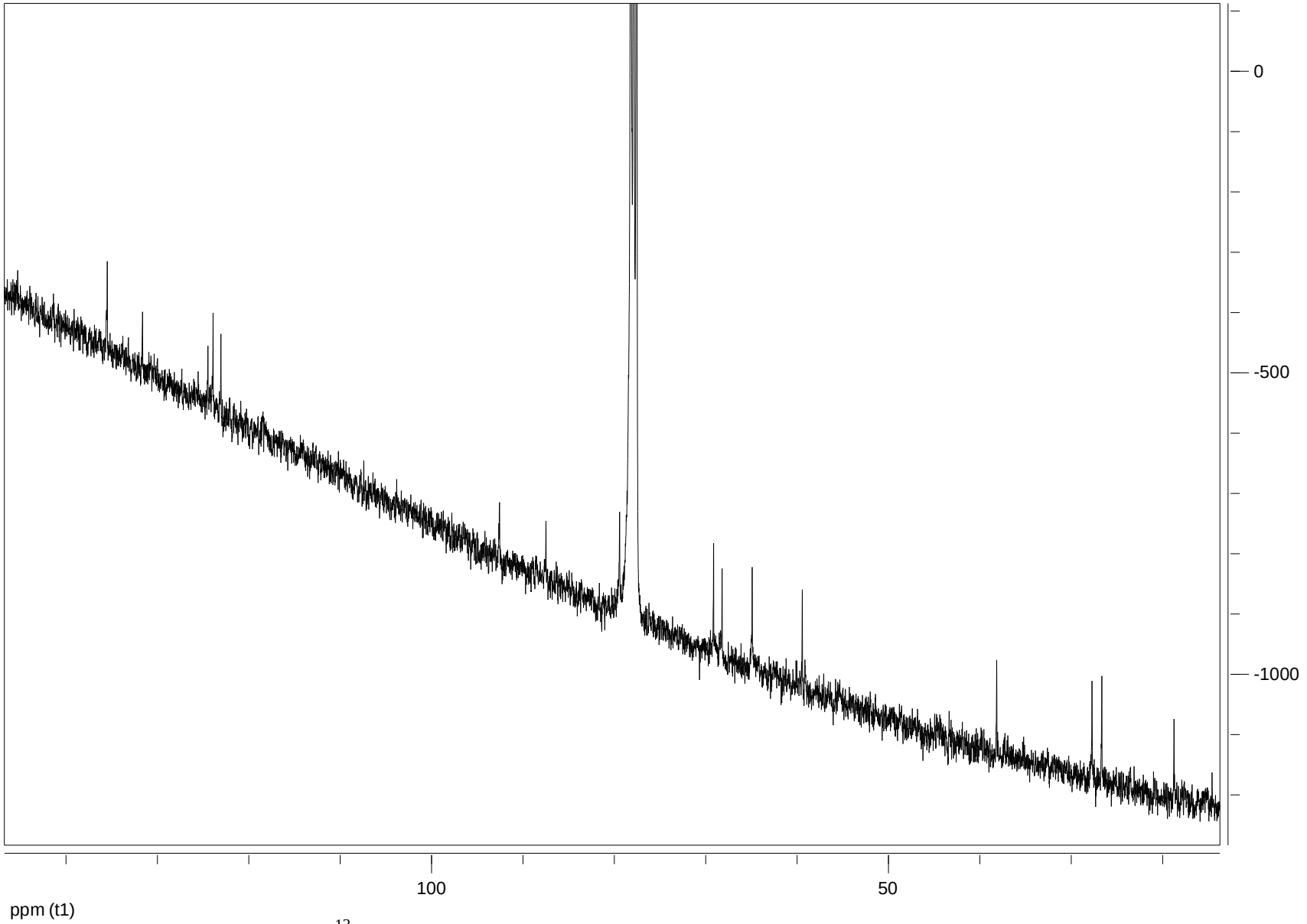


Figure 54. ^{13}C NMR spectrum of phomentrioloxin (**14**) recorded in $CDCl_3$ at 400 MHz

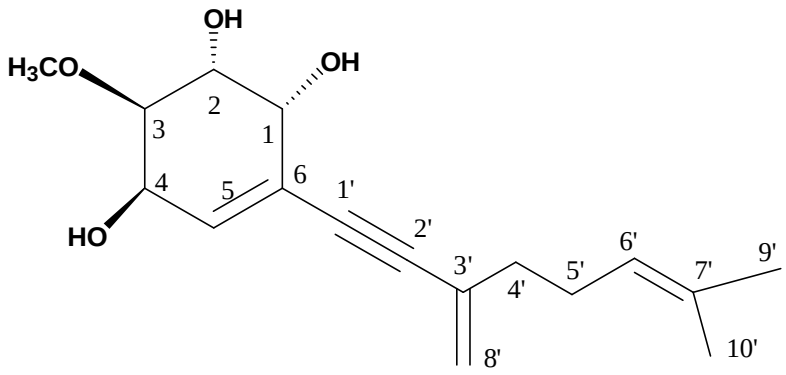


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Fig. 55. Structure of phomentrioloxin (**14**)

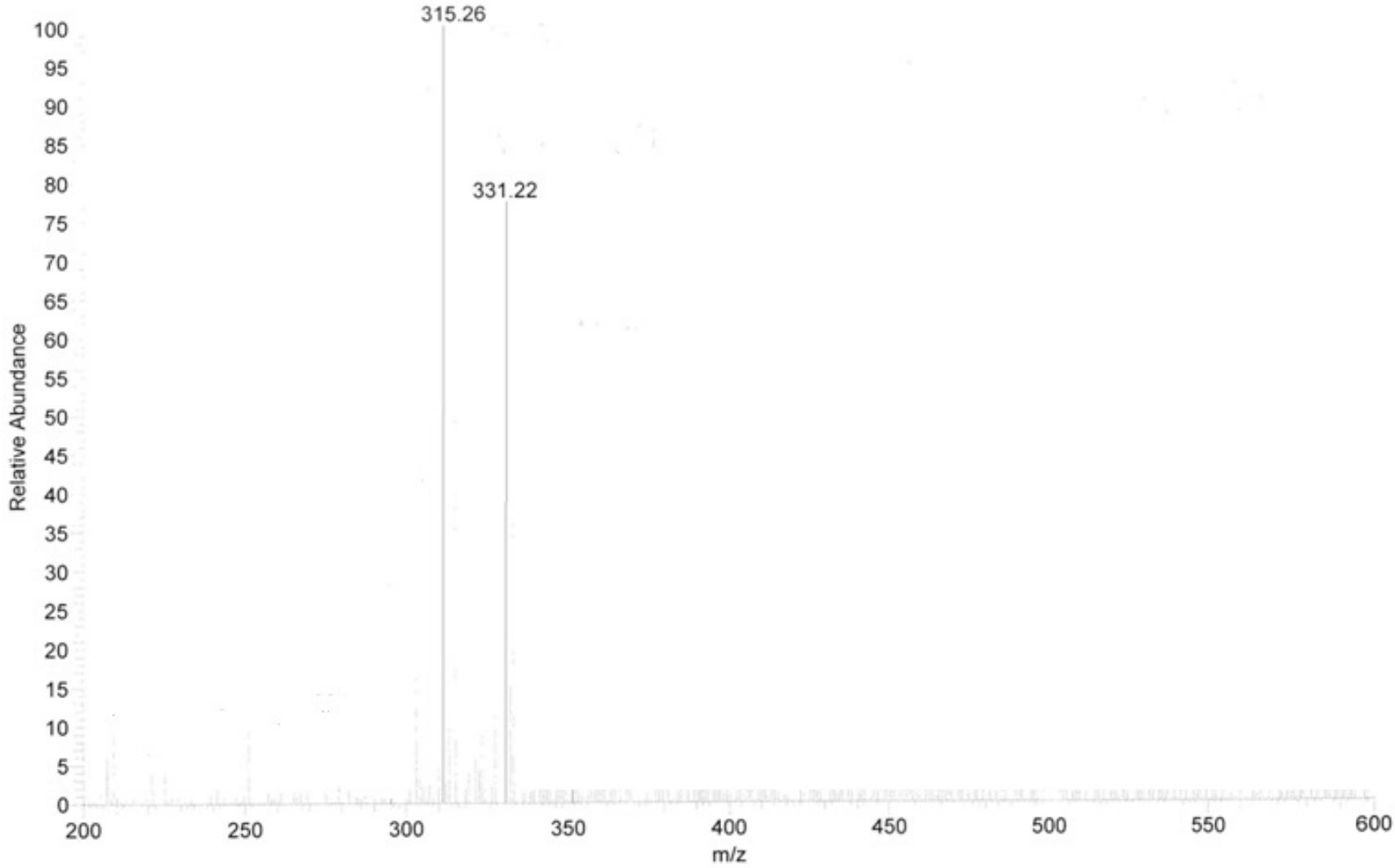

Figure 56. HR-ESIMS spectrum of phomentrioloxin (**14**) recorded in positive modality.

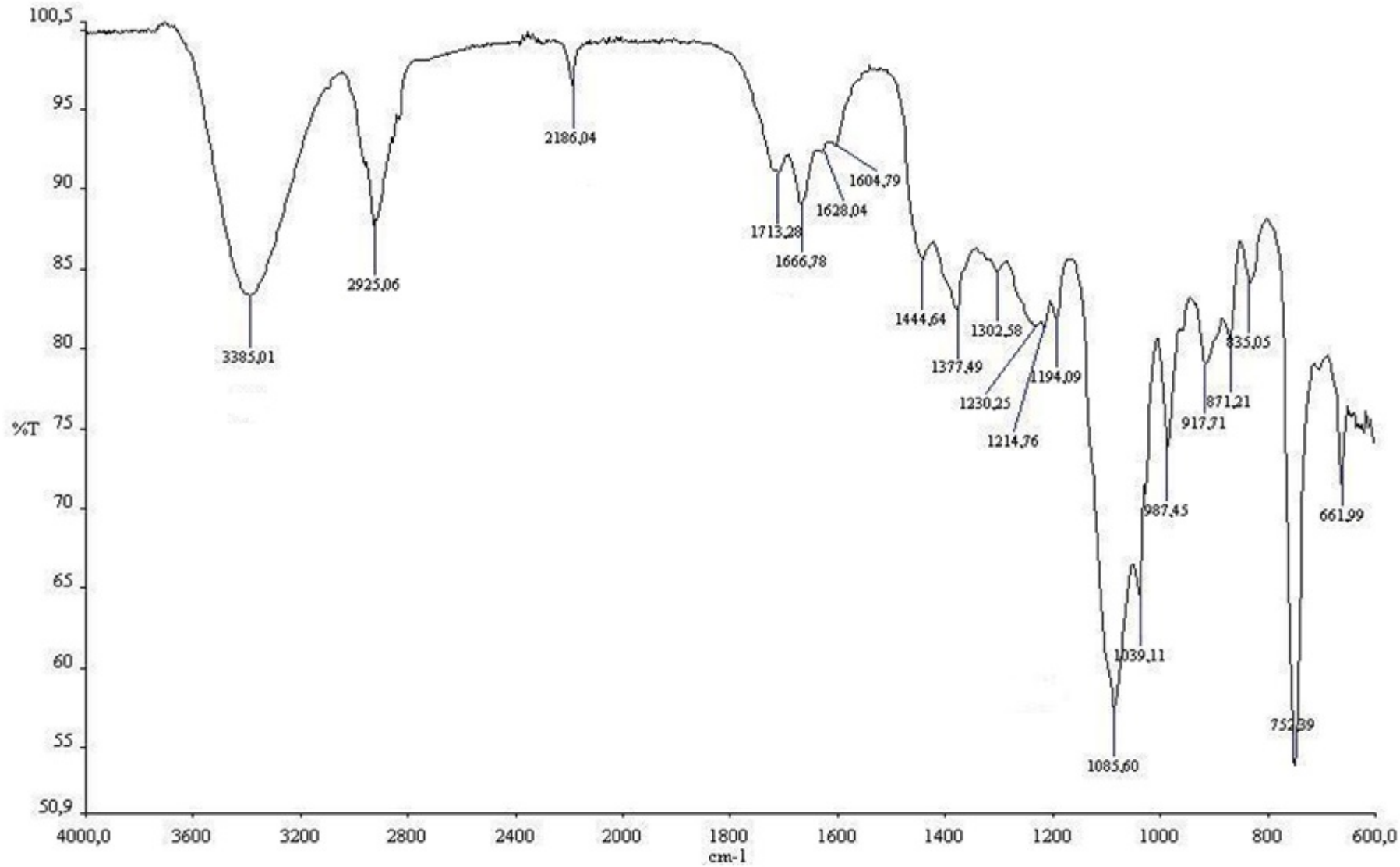

Figure 57. IR spectrum of phomentrioloxin (**14**) recorded as glass liquid.

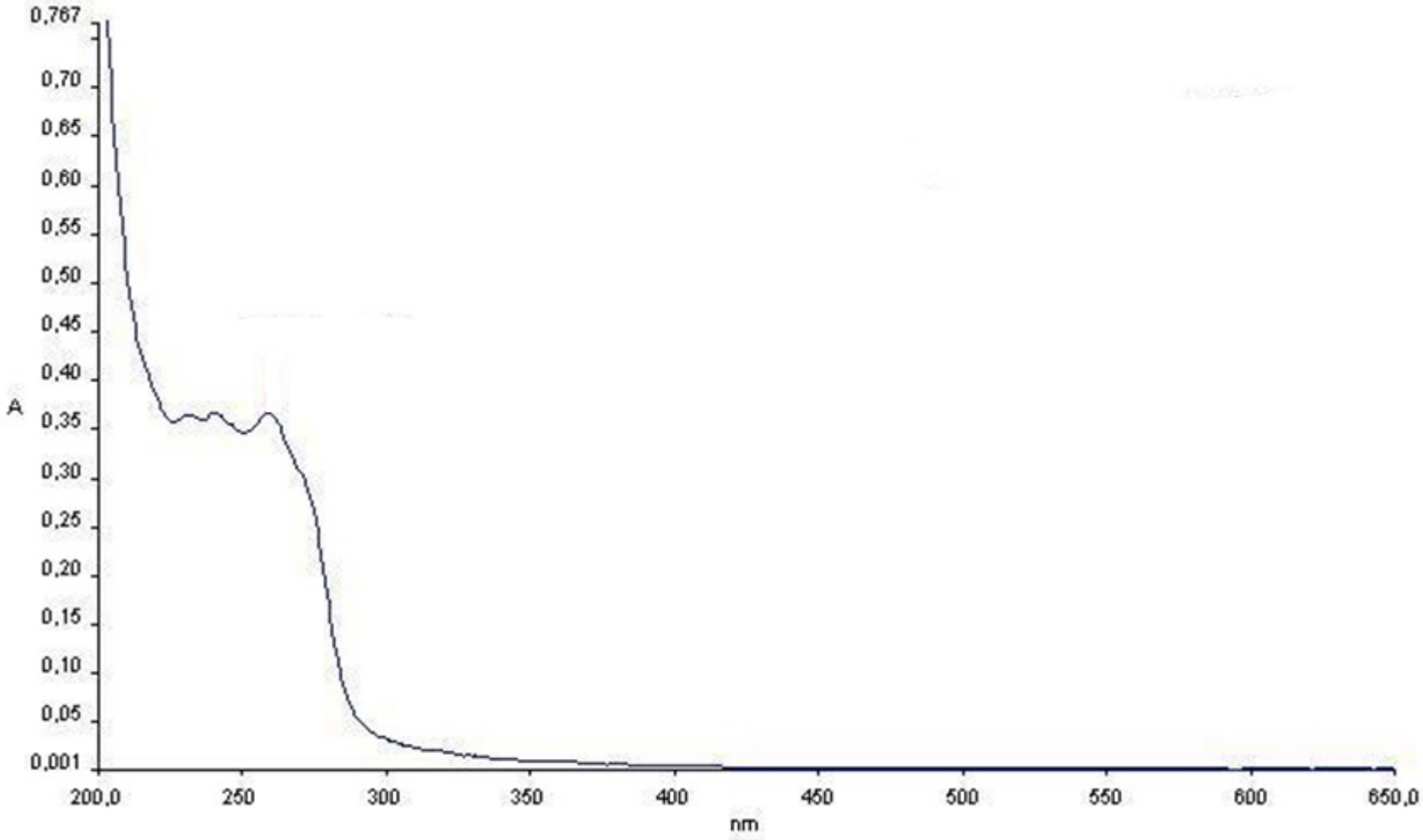

Figure 58. UV NMR spectrum of phomentrioloxin (**14**) recorded in MeCN solution.

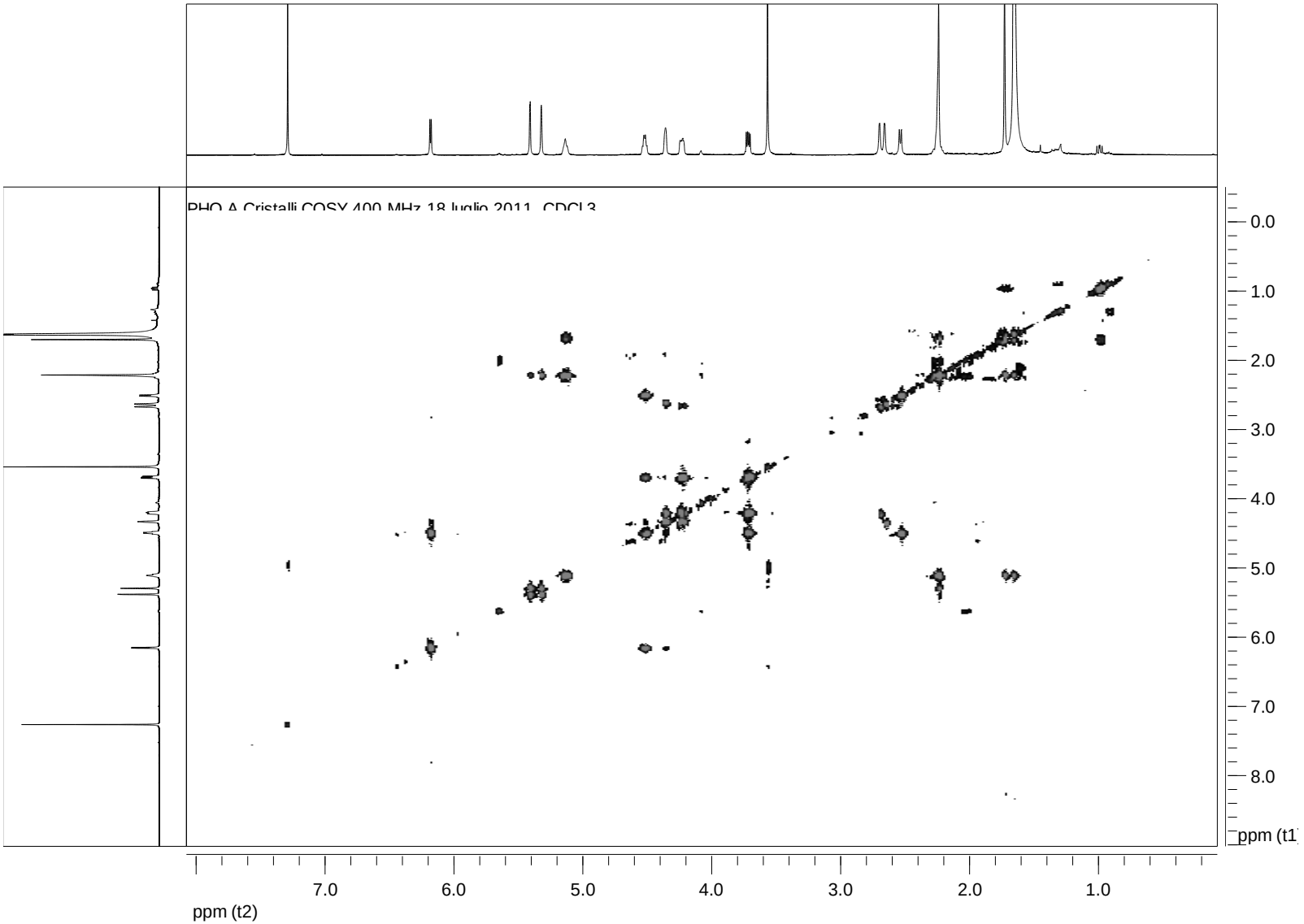

Figure 59. COSY spectrum of phomentrioloxin (**14**) recorded in $CDCl_3$ at 400 MHz.

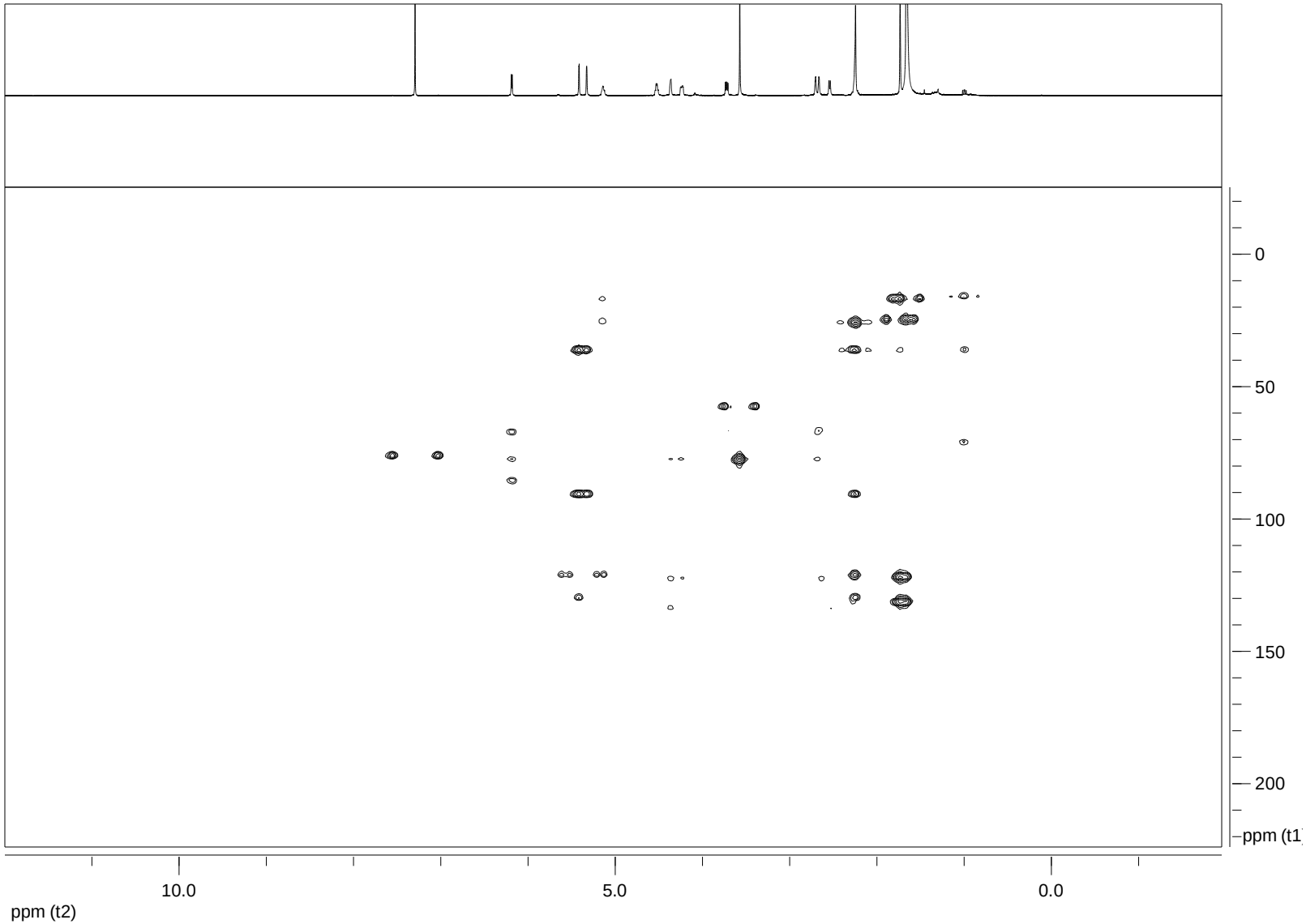

Figure 60. HMBC spectrum of phomentrioloxin (**14**) recorded in $CDCl_3$ at 400 MHz.

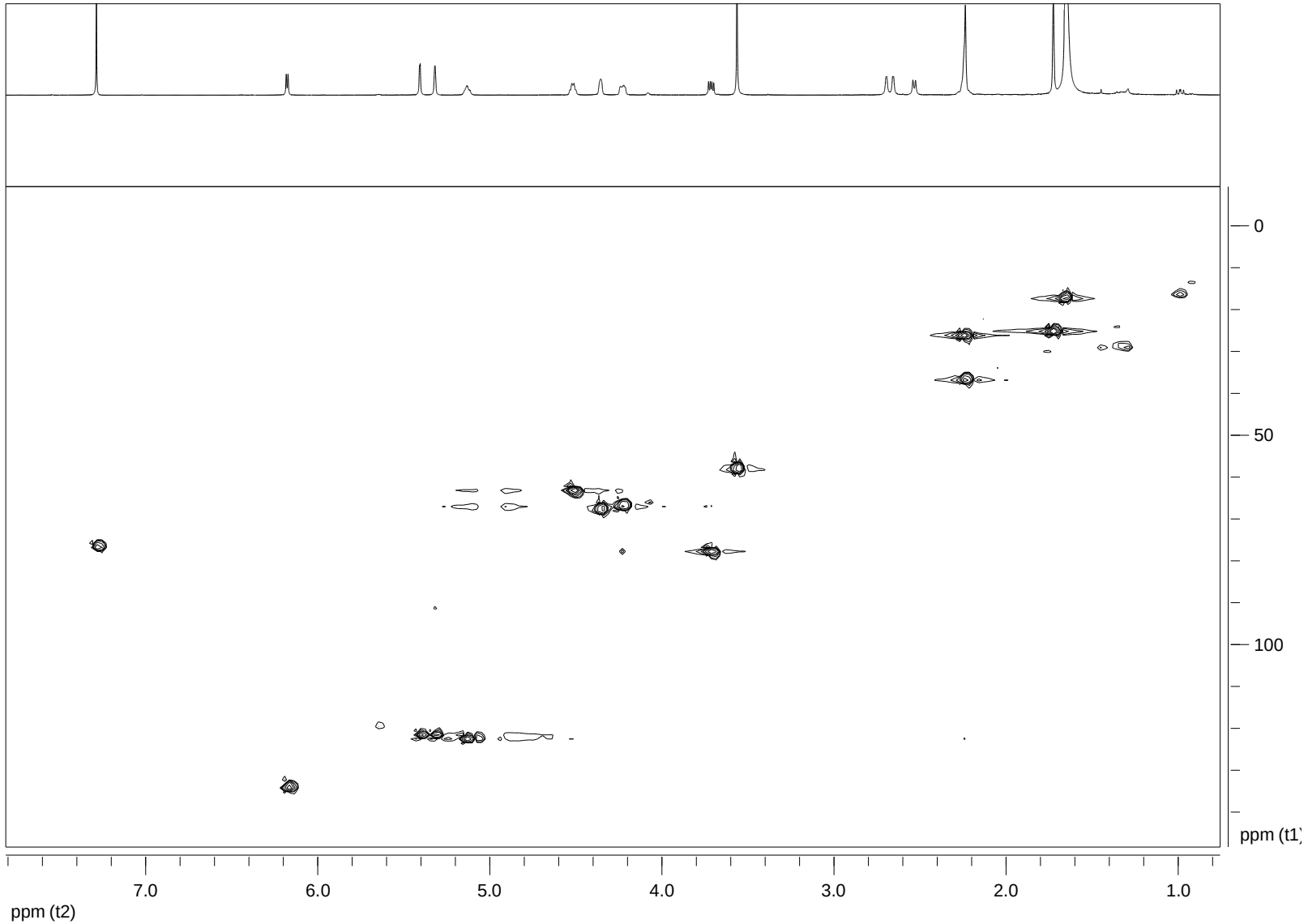

Figure 61. HSQC spectrum of phomentrioloxin (**14**) recorded in $CDCl_3$ at 400 MHz.

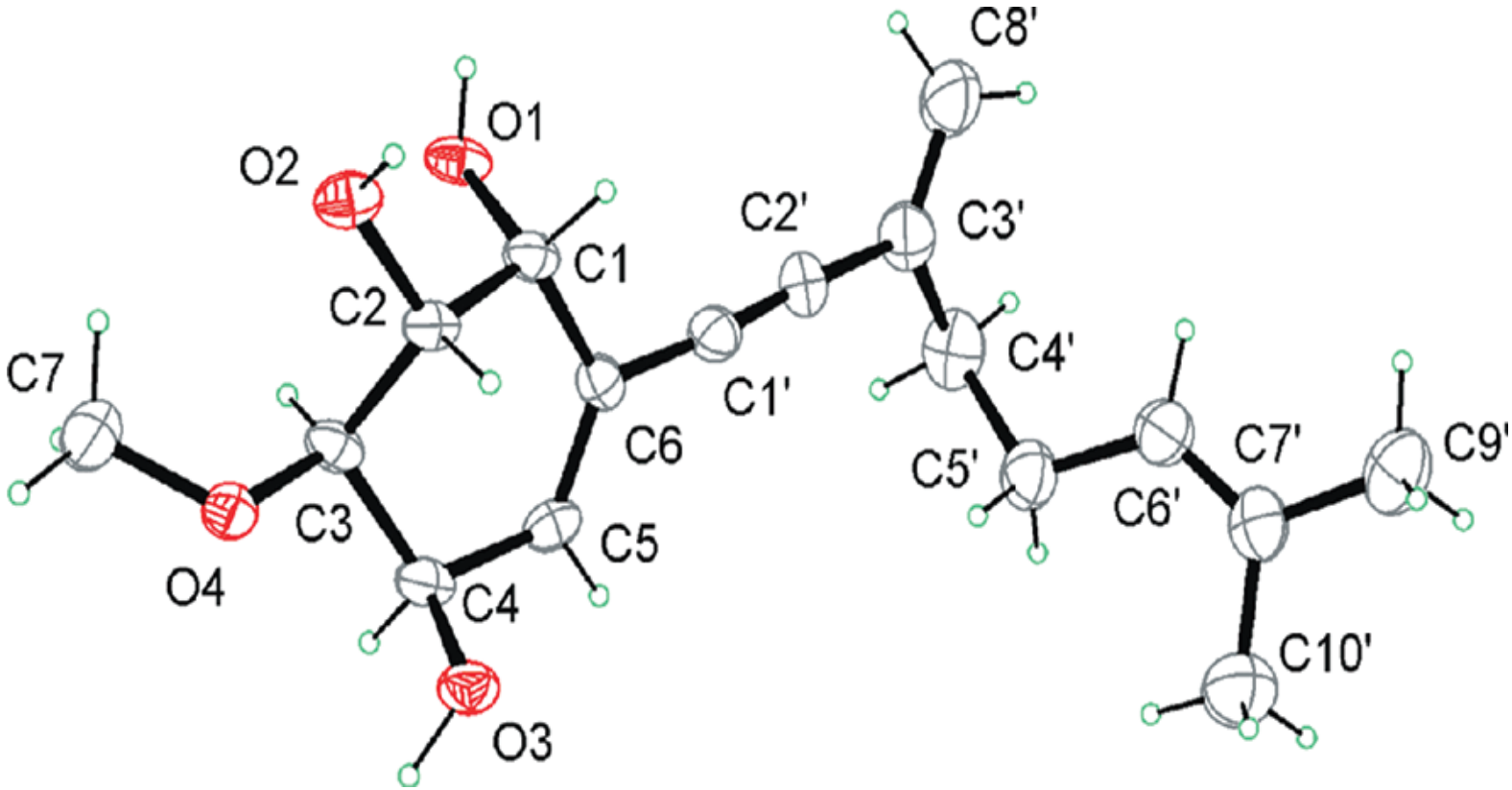

Figure 62. Ortep view of phomentrioloxin (**14**) showing atomic labeling

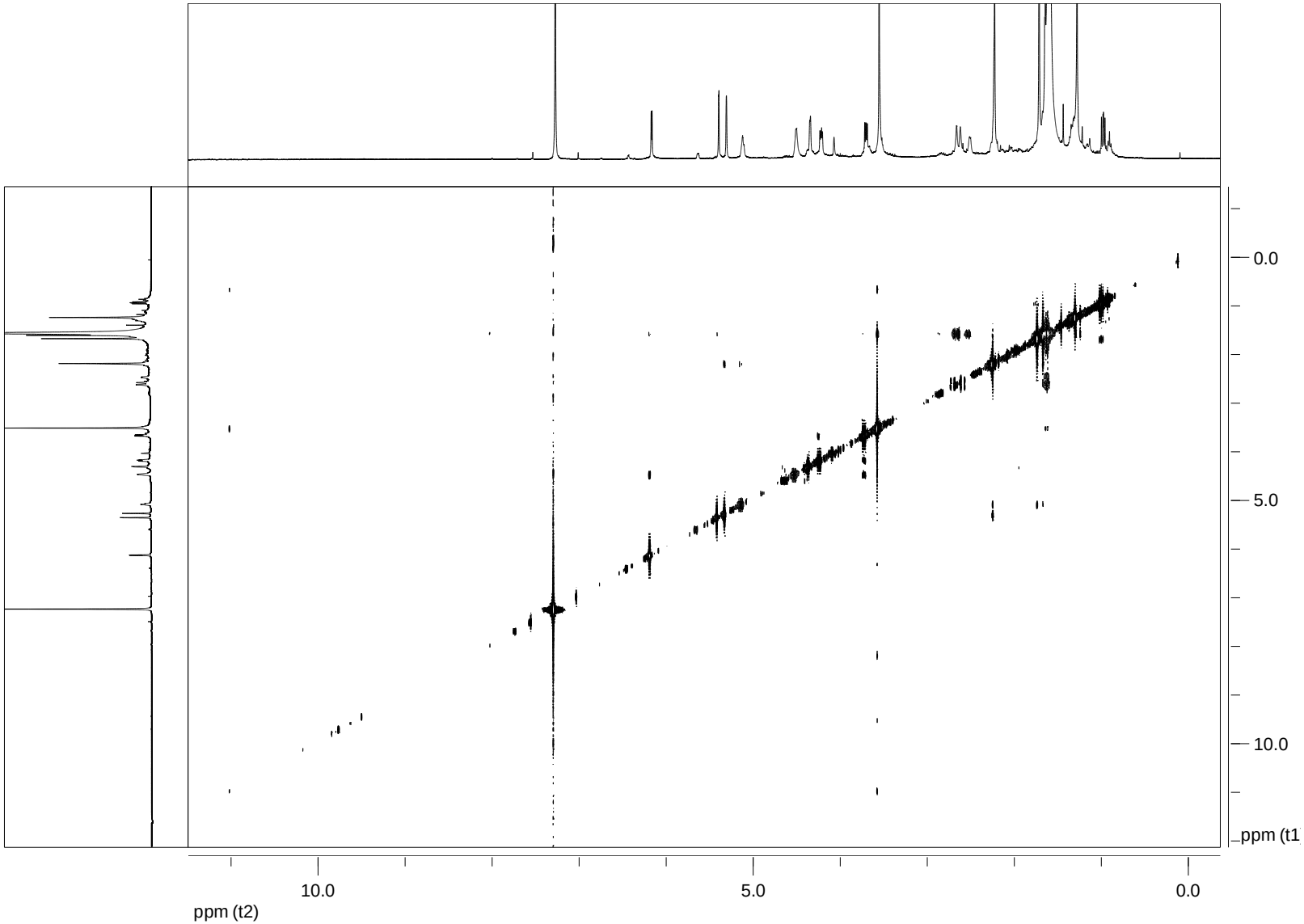

Figure 63. NOESY spectrum of phomentrioloxin (**14**) recorded in $CDCl_3$ at 400 MHz.