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Phytotoxins produced by *Alternaria sonchi*, a potencial mycoherbicide for biocontrol of *Sonchus arvensis*

Ph.D. THESIS

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1. INTRODUCTION

1.1. Weeds and their management

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 or ornamental crops. They provide competition for space, nutrients, water and light, although how seriously they will affect a crop depends on a number of factors.

The presence of weeds does not necessarily mean that they are competing with a crop, especially during the early stages of growth when each plant can find the resources it requires without interfering with the others. However, as the seedlings size increases, their root systems will spread as they each begin to require greater amounts of water and nutrients. Estimates suggest that weed and crop can co-exist harmoniously for around three weeks, therefore it is important that weeds are removed early on in order to prevent competition occurring. Weeds competition can have quite dramatic effects on crop growth. Distribution of weeds is determined by various environmental and biological characteristics. Human activities are mainly responsible for their regional patterns and have certainly played an important role in their spread. Plant species are also affected when their habitat are disturbed (Harlan and dieWelt, 1965). In fact, in a balanced and healthy ecosystem weeds do not exist. They originated only after humans disturbed the balance of natural ecosystems, and weeds have now become their integral components. In agroecosystem weeds have evolved due to continuous selection pressure imposed by humans, technological advancement, and/or

through agricultural practices. The role of humans in selecting crop plants vis-à-vis evolution of weeds is clear from the fact that over 40 percent of the world's total weed species belong to Asteraceae (sunflower family) and Poaceae (grass family), which happen to provide over half of world's food and food products. 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).

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, productions 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 tolerators, 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 know as competitive ruderals that grow rapidly and competition in them 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).

Such the typical plant species are *Cirsium arvense* (L.) Scop. (**Fig. 1**) and *Sonchus arvensis* L. (**Fig. 2**) (both from *Asteraceae*) commonly called Canada thistle and perennial sowthistle, respectively (Donald, 1990; Lemna and Messersmith, 1990).

Canada thistle is a persistent perennial weed that grows vigorously, forming dense colonies and spreading by roots growing horizontally that give rise to aerial shoots. 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).

Classified as a noxious weed in many states and provinces, 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. At high densities (27 shoots/m²), it has reduced spring wheat yields up to 45 percent in North Dakota. Perennial sowthistle is also a host of several economically important plant pests (Lemna and Messersmith 1990). A 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).

On the Canadian prairie, perennial sow-thistle is among the 15 most abundant weeds in annual crops. It was the most abundant weed in the biennial-perennial category in Manitoba between 1975-1978 (Thomas and Donaghy, 1991) with 39% of the cultivated fields infested with an average of 4.8 plants/m². Densities of 5 and 10 *S. arvensis* shoots/m² reduced canola

yields by 12 and 18% (Peschken, 1984). In Alberta, Saskatchewan and Manitoba rapeseed yield reductions were estimated at 6.7 million dollars annually. Densities of 3 to 27 stems/m² reduced spring wheat yields by 4.5 to 27% (Lemna and Messersmith, 1990). Perennial sow-thistle is readily eaten by cattle so vigorous stands are confined to ungrazed areas. There are two varieties of perennial sow-thistle in Canada that hybridize: var. *arvensis* and var. *glabrescens*. In some floras these are given species status, *S. arvensis* and *S. uliginosus* respectively. Both have a hairless lower stem, but in *S. arvensis* the upper stem and bracts have conspicuous gland-tipped hairs. *Sonchus arvensis* var. *arvensis* is most abundant in Ontario, Quebec and the Atlantic provinces. Var. *glabrescens* is most abundant on the prairies and extends north to Great Slave Lake.

Two annual sow-thistles also occur in Canada: *Sonchus asper* and *Sonchus oleraceus*. These have taproots but their flower heads are smaller, 1.5-2.5 cm in diameter. *S. asper* has unlobed leaves with weak spines. *S. oleraceus* has deeply lobed leaves that are almost spineless. Annual sow-thistles are an increasing problem. In 1987 they ranked 48th in abundance in Alberta cereal and oilseed crops. By 1997 they were 29th in the survey (Thomas *et al.*, 1998) and they are most serious in pulse crops.

Perennial sow-thistle seed germinates when the soil has warmed in the spring, weeks until the leaves are about 3 cm long and then forms a rosette. First year rosettes form vertical roots up to 2 m deep, produce vegetative buds from depths up to 50 cm and horizontal roots with a spread of 60-100 cm. The roots are mycorrhiza (Wein *et al.*, 1992). Bolting usually occurs in the second year when the rosette has 12-15 leaves. When cut during cultivation, root pieces as small as 1 cm can produce a flowering plant within a year.

Flowers are produced as a succession from early July until frost. They open 2-3 h before sunrise and close near noon, are insect pollinated and cross pollination is necessary for viable seed production. Each head produces about 30 seed and in oats produces about 3,000

seeds/plant. The seed, attached to a pappus, is dispersed by wind (about 10 m in a 16 km/h wind) and hooked pappus tips catch in fabric and animal fur. In Saskatchewan, 18 out of 20 seeds flew out of sight at average wind speeds of 15 km/h gusting to 22km/h. The pappus of all but one of 26 seeds was firmly attached at wind speeds averaging 7 km/hr and gusting to 22 km/h. The pappus of one seed became entangled on vegetation and fluttered in the wind for 15 minutes but did not separate from the seed (Peschken, 1984.).

Seed is also spread in hay. Seed viability is 70-90% with 85% emerging in the first year. Seedling survival is generally poor unless they are under litter or are irrigated. The thistle is moderately resistant to most broadleaf herbicides, but auxin type herbicides used on early vigorous growth can prevent flowering.

A combination of chemical and cultural controls is often more effective at reducing both crop competition and reproduction by both seed and root. The introduction of herbicide tolerant canola has reduced losses in this crop; but a serious problem remains in field peas and beans.

The effort to control weeds is as old as agriculture itself. Humans, however, were familiar with weeds even before the dawn of agriculture, as several aboriginal nomadic tribes suffered from allergies, hay fever, and other health problems caused by poisonous plants. Weeds management has progressed from bare hands to tools, to animal power, to machine power and finally to chemicals and integration of such powers. Weed management strategies have evolved with the advancement of agricultural technology, shifts in weed flora, and the formation of weed biotypes with herbicidal resistance (Kohli *et al.*, 2006).

Today chemical methods have largely replaced the other methods of weed management. Although fraught with accompanying problems of pollution of soil and groundwater, and toxicity of food products, herbicides are valuable and important tools that have provided major benefit to the production system (Buhler, 1999). However new and alternate options

would provide farmers with more flexibility for enhancing the effectiveness of the herbicides. These includes herbicidal mixtures, varietal mixtures, synergist, herbicide antidotes, breeding of herbicide-tolerant and more competitive crops, allelopathy and genetic engineering (Gressel, 1992, 2002).

1.2. Weed management strategies

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 removal 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.

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 use 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 that differ widely in respect to spectrum, 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 and 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 and Messersmith, 1990; Grekul *et al.*, 2005). These chemical 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 and Abouzeid 2006). Management of weeds, should, therefore, be achieved through strategies that do not affect the sustainability of agroecosystems and the life support system. Obviously, new compounds should be actually developed as herbicides against the composite weeds. The biological agents offer the advantage of being compatible with the environment, 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 vegetations 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 diverse environment. 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 and Abouzeid 2006). The identification and the biological and molecular characterization of microorganisms, useful as biocontrol agents or as producers of bioactive compounds, is of great interest for the modern and echo-compatible 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. 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 programmes. 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 effort to the agricultural biological production which is more and more present in the national and international markets.

In this respect these bioactive secondary metabolites could play an interesting role in the induction of disease symptoms (phytotoxins, antibiotics and phytohormones) 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) and in combination with low dose of herbicides and the phytopathogenic fungus, to develop integrated weed management strategy.

1.3. Fungal phytotoxins

Numerous studies have been conducted on the use of natural enemies such as insects, nematodes, bacteria and fungi for weed control, but particular interest has been directed to phytopathogenic fungi that could be applied with safety and simplicity. They have attracted attention due to the hazards they cause to the agricultural productivity of economic interest and the environmental damage for which they are responsible.

Plant pathogens are good sources of potent phytotoxins (Abbas and Duke 1995), as they usually kill tissues before they consume them. In spite of this, those that kill weed species have received relatively little attention in natural product herbicide discovery efforts, with a notable exception. Maculosin, produced by *Alternaria alternata*, a pathogen responsible for diseased spotted knapweed (*Centaurea maculosa* Lam.), is the first phytotoxin 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 and colleagues (1991).

Almost all fungal species produce phytotoxic metabolites. (Evidente and 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 culture and in their 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 are translocable within the plant. The virulence of the plant pathogen may depend on its capability to synthesize one or more toxins.

Fungal phytotoxins have facilitated advances in our understanding of numerous 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.

Fungal phytotoxins can be classified as host-selective or non-selective. Although it is difficult to find a clear line of demarcation, host selective toxins 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 is 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 l 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).

Phytotoxin production is sensitive to a number of diverse 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 vary considerably in their capacity for phytotoxin production (Berestetskiy, 2008). Microorganism strains are genetically unstable, and their storage or reinoculation may adversely affect the ability to produce toxins (Kale and 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 extracting toxin in advance of colonization and live as saprophytes from the dead substrate. Actually, leakage of cell constituents frequently occurs after the application of host-specific phytotoxins and non-host-specific phytotoxins.

1.3.1. Host-specific phytotoxins

This class of extracellular fungal metabolites is produced by plant-specific pathogens. Some compounds are so specific that they are only toxic to certain cultivars, e.g. maculosin, a cyclic dipeptide which is produced by *Alternaria alternata* and is host-specific to spotted knapweed (*Centaurea maculosa*). Similarly, bipolaroxin from *Bipolaris cyanodontis*. Shoemaker, a fungal pathogen of Bermuda grass (*Cynodon dactylon*), has been found to be host selective in low concentrations. At concentrations 20 times greater than that required to affect Bermuda grass, bipolaroxin causes phytotoxicity to wild oats, sugarcane and maize. (Saxena and Pandey, 2001)

Maculosin is the only host-specific phytotoxins for spotted knapweed in the true sense. Phomalairdenone is a new member of this group. This is produced by black crop species share common problem weed species, so host-specific toxin will be of little use and, commercially, it would be prohibitively expensive to develop and use different herbicides for each weed species, when compared to non-specific-toxins. (Saxena and Pandey, 2001).

1.3.2. Non-host-specific phytotoxins

Non-host-specific phytotoxins have a broader range of activity and applicability on weeds. Tentoxin, a by-product of *Alternaria alternata*, is a cyclic tetrapeptide causing phytotoxic damage to both monocotyledonous and dicotyledonous weed species. The mode of action of this secondary metabolite is the inhibition of CF1 ATPase activity (Steele *et al.*, 1978). Zinniol is produced by a number of *Alternaria* spp. and *Phoma macdonaldii* Boerma. It causes necrosis in tissues, probably through calcium regulation (Strobel and Sugawara 1986; Thuleau *et al.*, 1988).

Scopulariopsis candidus, *Cephalosporium* sp. and *Fusarium* sp. produce a potent phytotoxins, 1233A, which is inhibitor of 3-hydroxy-3-methylglutaryl co-enzyme A

synthetase (Greenspan *et al.*, 1987). *Ascochyta hyalospora*, the causal agent of leaf spot on lambsquarters, produce several phytotoxins: ascochyte, pyrenolide A and hyalopyrone. All three compounds exhibit phytotoxic activity to nine weed species, including *Chenopodium album* (lambsquarters), *Sida spinosa* L. (prickly sida), *Ipomea* sp. (morning glory) and *Sorghum halepense*. Recently, two phytotoxic nonenolides (viz. putaminoxin and pinolidoxin) produced by phytopathogenic *Phoma* and *Ascochyta* species have exhibited potent herbicidal activity (Evidente *et al.*, 1998b).

Fusaric acid is produced both by the virulent plant pathogen *Fusarium oxysporum* Schlechtend Fr. and by non-pathogenic fusarial species. Fusaric acid has been demonstrated to have herbicidal activity against several weed species, including jimsonweed and duckweed (Vesonder *et al.*, 1992).

1.3.3. Biological activities of fungal toxins

One of the major difficulties in studying and utilizing a given phytotoxin is its availability. This problem was overcome when European groups in 1970s succeeded in isolating and characterizing fusicoccin, a phytotoxin produced by *Fusicoccum amygdali* (Ballio *et al.*, 1964; Ballio 1977). This unleashed an unprecedented number of physiologists, biologists, chemists, pathologists, and agronomists in an attack on the mode of action, usefulness, and general biology of this phytotoxin. Each of the many new phytotoxins that has since been described provides a new target for a thorough, concerted chemical and biological investigation.

Besides their obvious role in the development of symptoms of certain plant diseases, phytotoxins also possess some unusual chemical and biological properties.

The effect of phytotoxins on plants is characterized by the appearance of specific symptoms; wilting and general growth suppression, as well as chloroses, necroses, and spotting of aerial portions are the most common. The reverse is also true: if a plant disease has symptoms described above, its causative agent conceivably forms phytotoxins, which play a role in the pathogenesis.

Reviews on phytotoxins, published in Russia, cover toxins produced by soil micromycetes (Berestetskiy *et al.*, 1976; Berestetskiy and Borovkov, 1979; Berestetskiy and Borovkov 1981) and causative agents of plant diseases from the genera *Alternaria* (Belyakova and Levkina, 1990), *Fusarium* (Bilai *et al.*, 1977), *Phytophthora*, *Verticillium* (Filippov *et al.*, 1980), *Phoma*, and *Ascochyta* (Uspenskaya and Reshetnikova, 1975).

As a rule, plants sensitive to a specific phytotoxin (genera, species, and even cultivars) fall within the range of the hosts of its producer. The spectrum of activities of a non-specific phytotoxin is not limited to phylogenetic specialization of the producer pathogen. If the list of

sensitive plants is still limited, e.g., to members of a certain family, the non-specific toxin is considered selective. (Berestetskiy, 2008)

Depending on the pathogenetic role, specific phytotoxins are divided into pathotoxins and vivotoxins. Pathotoxins (as a rule, at very low concentrations, of nanomolar to micromolar order) are prerequisite to induce plant infection by necrotrophic pathogens (certain species of *Alternaria*, *Cochliobolus*, *Drechslera*, etc.). Mutant strains of these fungi, incapable of synthesizing pathotoxins, lack virulence. (Berestetskiy, 2008)

By definition, vivotoxins are synthesized by pathogens in infected plant tissues; using appropriate concentrations, these toxins account for the emergence of certain symptoms of the disease. Vivotoxins are commonly non-selective. (Sock and Hoppe, 1999). A considerable number of phytotoxins are toxic to animals and/or microorganisms. Depending on the economic importance, they are classified with mycotoxins or antibiotics. For example, ascochyta, the phytotoxin produced by *Ascochyta pisi*, the causal agent of a leaf-spotting disease in pea, is structurally related to the potent mycotoxin citrinin and exhibits antifungal properties (Oku and Nakanishi, 1963; Lepoivre, 1982). The well-known antibiotic griseofulvin (one of its producers is *Penicillium griseofulvum*) is also phytotoxic (Berestetskiy and Borovkov, 1979).

Biological assays are used for both identifying phytotoxins in culture liquid and assessing phytotoxic activity of extracts or pure substances. In selecting biological assays, the biology and ecology of the fungus are taken into account. For example, if a phytotoxin originates in soil fungi or is causative agent of root rot diseases, the bioassay involves plant seedlings. The extent of growth suppression of roots treated with serial dilutions of the culture filtrate or the pure toxin is calculated using untreated roots as controls. If the symptoms of the disease caused by a phytotoxin producer involve leaves, a solution of the substance tested or the culture filtrate (5-20 μ l) is applied on to the leaf punctured by a needle.

The results (chloroses, necrotic spots) are read after 24-72 h. In order to reduce the requisite time for obtaining the results, isolated leaves (or parts thereof) may be placed into a moist chamber or onto the surface of water agar (Berestetskiy, 1982; Stierle *et al.*, 1992). Of course, the spectrum of bioassays is considerably broader than those described.

Thus, in recent years, effects of toxins have increasingly been studied in cultures of plant or animal cells. The use of several bioassays and diverse toxin concentrations likely increases the value of the results. In addition, it is desirable to assay new phytotoxins simultaneously for antibiotic and zootoxic activities (Cole *et al.*, 1986; Kohmoto, 1992).

1.3.4. Potential new herbicides

Microbially produced herbicidal compounds have relatively short lives, compared to synthetic, halogenated chemical structures. They are biodegradable and do not leave residues toxic to the environment. They are active in small quantities, compared to the high quantities of pesticides currently used. Thus, it can be generalized that significant contamination of food products, or the soil and water would be less likely with microbial compounds than with most synthetic herbicides used at the same rates (Saxena and Pandey, 2001).

Most microbial phytotoxins are water-soluble and non-halogenated compounds. They are also more benign toxicologically and environmentally, compared to synthetic herbicides. They have built-in species-selectivity, perhaps due to their isolation from host-specific plant pathogens and weed hosts. This is a highly desirable property, as avoidance of injury to crop plants is a goal of synthetic herbicide development programs (Saxena and Pandey, 2001).

Microbial phytotoxins have a limited shelf-life as compared to synthetic chemicals. Finding new herbicides with a new site of action is most important, since the rate of appearance of weeds that have evolved resistance to synthetic herbicides has increased logarithmically and market niches for currently exploited sites are reaching saturation (Saxena and Pandey, 2001).

Microbial phytotoxins are used as tools for envisaging new molecular sites of action not discovered by the traditional approaches of herbicide discovery (Cutler 1991). Moreover, there is little overlap between the sites of action of phytotoxins and those of traditional or commercial herbicides. (**Table 1**). The newer sites would be useful in overcoming the current herbicide resistance problems encountered in weeds. These attributes of microbial phytotoxins have been found to satisfy the complex set of questions put forth by crop protection research groups; and this has influenced research by institutions and industry (Ayer *et al.*, 1986;

Kenfield *et al.*, 1989). With advances in chemical technology and biotechnology, this strategy is becoming less time consuming.

Traditionally most investigators were concerned with the isolation, characterization and mode of action of phytotoxins from plant pathogens of crop plants.

Numerous surveys were carried out to find pathogens of *C. arvensis* (Berestetskiy, 1997; Leth and Andreasen, 1999; Bailey *et al.*, 2000) and, to a lesser extent, of *S. arvensis* (Berestetskiy and Smolyaninova, 1998).

Phytopathogenic fungi belonging to the genus *Ascochyta* are responsible for several diseases, that cause necrotic lesions on leaves and stems (Melnik, 1971).

Some *Ascochyta spp.* have also been proposed as mycoherbicides for the biological control of noxious weeds, i.e.: *A. caulina* against *Chenopodium album* (Netland *et al.*, 2001), or *A. cypericola* against *Cyperus rotundus* (Upadhyay *et al.*, 1991). The ability of many of these pathogens to produce phytotoxins has been ascertained and their involvement in symptoms appearance has been discussed (Evidente *et al.*, 1993a,b; Strange, 1997). Recently, three novel toxins have been purified and identified from the liquid culture of *A. caulina* and proposed as natural herbicides to be utilized in addition to or as an alternative to the use of the pathogen (Evidente *et al.*, 1998a, 2000; Vurro *et al.*, 2001).

Ascochyta sonchi (Sacc.) Grove is a natural pathogen isolated from necrotic leaves of sowthistle (*Sonchus arvensis* L.) (Evidente *et al.*, 2004).

A new phytotoxic enol tautomer of 4-pyridylpyruvic acid, named ascosonchine, was isolated from the culture filtrate of *Ascochyta sonchi* (Evidente *et al.*, 2004).

Ascasonchine (**1**; **Fig. 3**), characterised as (Z)-2-hydroxy-3-(4-pyridyl)-2-propenoic acid by spectroscopic methods, showed selective herbicidal properties, that are not associated with antibacterial, antifungal or zootoxic activities (Evidente *et al.*, 2004).

A simple and sensitive method has been developed for the rapid quantitative analysis of ascosonchine based on HPLC with UV detection. The toxin content in culture filtrates of different strains of *A. sonchi* was measured. Toxin production was compared with the virulence on the host plant of each strain to determine if the most virulent strains could be simply selected by choosing the best toxin producers. The results obtained do not support this approach. The same HPLC method was also applied to quantify toxin production under different fungal growth conditions, in order to achieve the highest toxin production (Evidente *et al.*, 2006).

Two of the strains analysed, that don't produced ascosonchine (C-177 and S-9) was reclassified as *Phoma exigua* var. *exigua*. (Cimmino *et al.*, 2008). It was demonstrated that the above two strains, grown in liquid and solid cultures, produced *p*-hydroxybenzaldehyde, cytochalasins B, F, Z2 and Z3, and deoxaphomin (2, 3, 4, 5, 6 and 7, **Fig. 4**). When assayed on the leaves of both *C. arvensis* and *S. arvensis*, *p*-hydroxybenzaldehyde was inactive, whereas deoxaphomin demonstrated the highest level of toxicity on leaves of *S. arvensis*. Cytochalasin Z2 appeared to be the less toxic cytochalasin on both plants according to the lack of the secondary hydroxyl group on C-7 (Cimmino *et al.*, 2008).

Stagonospora cirsi, a fungal pathogen isolated from *C. arvensis* and proposed for its biocontrol, produces phytotoxic metabolites in liquid and solid cultures. Recently, the main metabolite, stagonolide A (8, **Fig. 5**), with interesting phytotoxic properties, was isolated from a liquid culture and characterized as a new nonenolide (Yuzikhin *et al.*, 2007). Five new nonenolides, named stagonolides B-F (9, 10, 11, 12, 13, **Fig. 5**), were isolated from solid culture and characterized using spectroscopic methods. When tested by a leaf disk puncture assay at a concentration of 1 mg/ml, these compounds showed no toxicity to *C. arvensis* and *S. arvensis*, whereas stagonolide A was highly toxic. Stagonolide A and stagonolide C were

weakly toxic to *Colpoda steinii*, a protozoan, when tested at 0.05 mg/ml, with the other stagonolides non-toxic (Evidente *et al.*, 2008a).

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 (14, 15, 16 and 17, Fig. 6), previously isolated from *Paraphaeosphaeria* sp., a fungus separated from the horse mussel (Tsuda *et al.*, 2003). Leaf disk-puncture assays at 1 mg/ml of stagonolides H-I and modiolide A were phytotoxic to *C. arvensis*. Only stagonolide H inhibited chicory seedling root growth. The most potent toxin, stagonolide H, indicated selectivity when tested on leaves of eight different plants. Canada thistle was most sensitive to the compound (Evidente *et al.* 2008b).

Considering that some pathogens of this perennial weeds produced nonenolides and cytochalasins, a structure-activity relationships study was conducted assaying 15 natural analogues and derivatives belonging this two groups of organic compounds. The toxic nonenolides (stagonolide A, putaminoxin, pinolidoxin) and cytochalasins (deoxaphomin, cytochalasins A, B, F, T, Z2 and Z3) were isolated from phytopathogenic *Stagonospora*, *Phoma* and *Ascochyta* spp. (Berestetskiy *et al.*, 2008).

Among the 15 compounds tested, stagonolide A and deoxaphomin proved to be the most phytotoxic to *C. arvensis* and *S. arvensis* leaves, respectively. The tested phytotoxic nonenolides were stronger inhibitors of photosynthesis in *C. arvensis* leaves than cytochalasins A and B. Stagonolide A had less effect on membrane permeability in *C. arvensis* leaves than cytochalasin B. Significant changes of light absorption by *C. arvensis* leaves in visible and infrared spectra were caused by stagonolide A. The functional groups and the conformational freedom of the ring, appear to be important structural features for the nonenolides toxicity, whereas and the presence of the hydroxy group at C-7, the functional

group at C-20 and the conformational freedom of the macrocyclic ring are important for the cytochalasins toxicity (Berestetskiy *et al.*, 2008).

Also *Phyllosticta cirsii*, a fungal pathogen isolated from diseased *C. arvense* leaves was evaluated as a biocontrol agent of this noxious perennial weed, and was found produce different phytotoxic metabolites with potential herbicidal activity when grown in liquid cultures (Evidente *et al.*, 2008c).

Phyllostictines A-D (**18**, **19**, **20** and **21**, **Fig. 7**), four novel oxazatricycloalkenones, were recently isolated from this pathogen and chemically and biologically characterized. Structure-activity relationship showed that the size and functionalities of macrocyclic ring are features important for the phytotoxicity, with the β -lactone appeared to be unessential (Evidente *et al.*, 2008d). To support the potential use of phyllostictine A as a natural herbicide, toxin production has been studied using different media and cultural conditions. The toxin content in the crude extracts has been determined by using a HPLC method set up for this purpose. Furthermore, its phytotoxicity has been evaluated on tobacco protoplasts by flow cytometric analysis, and on *C. arvense* protoplasts, by fluorescence microscopy. The pure metabolite proved to have rapid dose-dependent toxic effects on host and non host plant protoplasts (Zonno *et al.*, 2008).

Further purification of the same organic extract provided two other metabolites, named phyllostoxin and phyllostin (**22** and **23**, **Fig. 8**), 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. This is not surprising, considering the noteworthy structural differences between the two compounds, suggesting the

presence of active functional groups in phyllostoxin not present in the other metabolite (Evidente *et al.*, 2008c).

Recently, the fungus *Alternaria sonchi* has been evaluated as a possible biocontrol agent of sowthistle (Gannibal *et al.*, 2006).

As also reported at paragraph 1.3, species belonging to the genus *Alternaria* are known to produce bioactive metabolites, including non-host phytotoxins e.g.: solanapyrones isolated by cultures of *A. solani*, the causal agent of early blight of tomato and potato (Ichara *et al.*, 1983), dextrusins, ciclodepsipeptides, isolated from *A. brassicae*, which causes diseases on numerous oil-yielding, vegetable, condiment, ornamental, and wild and some cultivated and wild non-cruciferous plants (Tewari and Bains, 1997). Brefeldin and α,β -dehydrocurvularin were isolated from *A. zinniae* (Vurro *et al.*, 1998) and several phytotoxins belonging to different group of natural compounds including toxic tetramic acid, dibenzo[a]pyrones moiety containing compounds, and alternatoxins I and II (Cole and Cox, 1981; Turner and Aldridge, 1983).

Considering the interest for bioactive metabolites produced by weed pathogens as sources of novel natural herbicides, it seemed interesting to investigate the production of toxins by *Alternaria sonchi*.

2. OBJECTIVES

The present thesis has different objectives all finalized to the identification of phytotoxic compounds produced in solid cultures by *Alternaria sonchi*, a fungal pathogen isolated from *Sonchus arvensis* and proposed as biocontrol agent of this noxious perennial weed.

1. The first aim is the isolation and the identification of the fungus. These studies were conducted by the research group of Dr. Alexander Berestetskiy , All Russian Institute of Plant Protection, in Saint Petersburg.
2. The second aim of the present thesis was to isolate from solid culture of *A. sonchi*, one or more metabolites with phytotoxic activity, using common techniques for their extraction (solid-liquid) and for chromatographic purification (CC and TLC) .
3. The third aim of the present thesis is to characterize by spectroscopic methods (IR; UV; ^1H and ^{13}C NMR; MS), the phytotoxins isolated from *A. sonchi*.
4. The fourth aim is the biological characterization of the phytotoxins isolated as potential herbicides, carried out in collaboration with the plant pathologist group.

3. MATERIALS AND METHODS

3.1. Fungus

The fungus *A. sonchi* Davis was isolated from diseased leaves of *S. arvensis* by Dr. Alexander Berestetskiy and monoconidial isolate (S102) was deposited in the culture collection of All-Russian Research Institute of Plant Protection, Pushkin, Saint-Petersburg, Russia. The isolate was maintained in sterile tubes containing potato-dextrose-agar (PDA).

3.2. General Procedures

Optical rotation was measured in CHCl_3 solution on a JASCO (Tokyo, Japan) P-1010 digital polarimeter.

IR spectra were recorded as glassy film on a Perkin-Elmer (Norwalk, CT, USA) Spectrum One FT-IR Spectrometer and UV spectra was taken in MeCN solution on a Perkin-Elmer Lambda 25 UV/Vis spectrophotometer.

^1H spectra were recorded at 600, 400 MHz, in CDCl_3 on Bruker (Kalsrhue, Germany) spectrometers. ^{13}C NMR spectra were recorded at 150, 100 and 75 MHz, in the same solvent and using the same instruments. The same solvent was used as internal standard. Carbon multiplicities were determined by DEPT (Distortionless Enhancement by Polarization Transfer) experiment. DEPT, COSY-45 (Correlated Spectroscopy), HSQC (Heteronuclear Single Quantum Correlation), HMBC (Heteronuclear Multiple Quantum Correlation) and NOESY (Nuclear Overhauser Effect Spectroscopy) experiments (Berger and Braun 2004) were performed using Bruker microprograms. Chemical shifts are in δ (ppm).

Coupling constants (J) are in Hertz. The following symbols were used: s =singlet; d : doublet; dd : double doublet; q : quartet.

ESI (ElectroSpray Ionization) and HRESI MS (Hight resolution ElectroSpray Ionization Mass Spectroscopy) spectra were recorded on Waters Micromass Q-TOF Micro and Agilent

1100 coupled to a JOEL AccuTOF (JMS-T100LC) (Milford, MS, USA) instruments. EI MS spectra were taken at 70 eV on a QP 5050 Shimadzu spectrometer.

Analytical and preparative TLC were performed on silica gel (Kieselgel 60 F254, 0.25 and 0.50 mm, respectively, Merck, Darmstadt, Germany) or reverse phase (Whatman, KC18 F254, 0.20 mm, Maidstone, UK) plates; the spots were visualized by exposure to UV light or by spraying first with 10% H₂SO₄ in methanol and then with 5% phosphomolybdic acid in ethanol, followed by heating at 110°C for 10 min. Column chromatography was performed on silica gel (Merck, Kieselgel 60, 0.063-0.200 mm).

4. EXPERIMENTAL

4.1. Production, extraction and purification of alternethanoxins A and B (24-25).

A. sonchi was grown on autoclaved pearl barley in ten 1000-ml Erlenmeyer flasks (pearl barley 100 g, water 60 ml) for 21 days in the darkness. Fungal metabolites were extracted from dry mycelium according to a slightly modified protocol of Evidente *et al.* 2002 (Evidente *et al.*, 2002). The dried material was extracted with the mixture acetone-2% NaCl (1:1, 2 l). The suspension obtained was centrifuged at 10000g for 40 min. The same protocol has been repeated two times on solid phase using the same volume of the mixture acetone-2% NaCl (1:1). The extract were combined and after acetone evaporation, the aqueous residue was lyophilized. The residue was dissolved in 500 ml of distilled water and extracted with EtOAc (3x500 ml). The organic extracts were combined, dried (Na₂SO₄) and evaporated under reduced pressure yielding a brown oily residue (975 mg). The organic extract, showing high phytotoxicity, was purified by silica gel column chromatography eluted with the CHCl₃-*i*-PrOH (9:1, v/v), to give 11 groups of homogeneous fractions (**Scheme 1**). Fractions were tested for bioactivity against *S. arvensis* as described below and those showing phytotoxicity were further purified. The residue (174.7 mg) of the fourth fraction was purified by silica gel column, eluted with CHCl₃-*i*-PrOH (95:5, v/v), to yield 5 fractions. The residue (88 mg) of the second fraction was purified by preparative TLC on silica gel [eluent CHCl₃-*i*-PrOH (95:5, v/v)], to yield 8 fractions. The residue (51 mg) of the sixth fraction appeared to be homogeneous yellow solid, which was named alternethanoxin A (**24**, *R_f* 0.38; 51.0 mg; **Fig. 9**). The residue of the fourth fraction was purified by preparative TLC on reverse phase [eluent EtOH-H₂O (6:4, v/v)] to yield an amorphous solid, which named alternethanoxin B (**25**, *R_f* 0.47; 2.2 mg; **Fig. 9**; **Scheme 1**).

4.1.1. Alternethanoxin A (24).

Alternethanoxin A (**24**, **Fig. 9**) obtained as an amorphous solid, had : $[\alpha]_{25}^D -16^\circ$ (*c* 0.2); IR ν_{\max} 3341, 1697, 1635, 1583, 1515, 1291 cm^{-1} ; UV λ_{\max} (log ϵ) nm 381 (sh); 299 (3.82); 241 (4.07); ^1H and ^{13}C NMR spectra: see **Table 2**; HRESIMS (+) m/z 627 $[2\text{M}+\text{Na}]^+$, 325.0701 $[\text{C}_{16}\text{H}_{14}\text{NaO}_6 \text{ calcd. } 325.0688, \text{M} + \text{Na}]^+$, 287 $[\text{M}-\text{Me}]^+$.

4.1.2. Alternethanoxin B (25).

Alternethanoxin B (**25**, **Fig. 9**) obtained as an amorphous solid, had : $[\alpha]_{25}^D -32.5^\circ$ (*c* 0.1); IR ν_{\max} 3232, 1688, 1656, 1608, 1589, 1519, 1291, 1259 cm^{-1} ; UV λ_{\max} (log ϵ) nm 381 (3.6), 294 (3.8), 262 (4.4), 237 (4.3); ^1H and ^{13}C NMR spectra: see **Table 3**; HRESI MS (+) m/z 623 $[2\text{M}+\text{Na}]^+$ 323.0541 $[\text{C}_{16}\text{H}_{12}\text{NaO}_6 \text{ calcd. } 323.0532, \text{M}+\text{Na}]^+$.

4.1.3. Triacetylalternethanoxin A (26).

Alternethanoxin A (**24**, 10.0 mg) was acetylated with acetic anhydride (70 μl) and pyridine (70 μl), at room temperature overnight. The reaction was stopped by addition of MeOH, and evaporated by a N_2 stream. The residue (11.0 mg) was purified by preparative TLC on silica gel [(eluent CHCl_3 -*i*-PrOH (98:2, v/v)], yielding the triacetyl derivative of alternethanoxin A (**26**, **Fig.10**) as an amorphous solid (*R_f* 0.56, 8.0 mg). It had: $[\alpha]_{25}^D -15^\circ$ (*c* 0.2); IR ν_{\max} 1770, 1724, 1670, 1620, 1575, 1433, 1176 cm^{-1} ; UV λ_{\max} (log ϵ) nm 287 (sh); 253 (3.93); ^1H NMR, δ : 7.82 (1H, d, $J=7.7$, H-8), 7.50 (1H, dd, $J=8.0, 7.7$, H-9), 7.40 (1H, d, $J=8.0$, H-10), 6.86 (2H, each s, H-3 and H-6) 3.72 (3H, s, OMe), 2.40 (3H, MeCO), 2.02 (3H, MeCOO), 1.95 (6H, 2xMeCOO); ^{13}C NMR, δ : 188.4 (MeCO), 168.8 (2xMeCOO), 168.5 (MeCOO), 165.9 (C-7), 150.5 (2C, s, C-4 and C-5), 147.6 (C-1), 144.2 (2C, s, C-6a and C-10a), 136.2 (C-10b), 130.6 (C-2), 129.0 (d, C-9), 127.3 and 127.2 (2C, d, C-8 and C-10),

122.0 (2C, d, C-3 and C-6), 52.5 (OMe), 21.55 (MeCOO), 20.5 (3xMeCOO); ESIMS (+) m/z : 879 [2M+Na]⁺, 451[M+Na]⁺.

4.1.4. Alternethanoxin A dimethyl ether (27).

To alternethanoxin A (**24**, 4.0 mg), dissolved in MeOH (0.5 ml), was added an ethereal solution of diazomethane. The reaction was carried out overnight at room temperature in the dark. The reaction was stopped by evaporation under N₂ stream. The residue (4.2 mg) was purified by preparative TLC on silica gel [(eluent petrol-Me₂CO (8:2, v/v)], yielding alternethanoxin A dimethyl ether (**27**, **Fig.11**) as an amorphous solid (R_f 0.31, 2.0 mg). It had: $[\alpha]_{25}^D$ -17 (c 0.2); IR ν_{\max} 2923, 1721, 1628, 1600, 1574, 1464, 1277 cm⁻¹; UV λ_{\max} (log ϵ) nm: 337 (sh); 285 (3.84); ¹H NMR, δ : 12.95 (OH, s), 7.61 (1H, d, J = 7.8, H-8), 7.36 (1H, dd, J = 7.8, 7.7, H-9), 7.11 (1H, d, J = 7.7, H-10), 6.46 (1H, s, H-3) and 6.05 (1H,s, H-6), 3.74 (3H, s, OMe), 3.72 (3H, s, OMe), 3.31 (3H, s, OMe), 2.29 (3H, s, MeCO); ESIMS (+) m/z : 683 [2M+Na]⁺, 353 [M+Na]⁺.

4.1.5. (S)- α -Methoxy- α -trifluorophenylacetate (MTPA) ester of alternethanoxin A (28).

(*R*)-(-)-MPTA-Cl (20 μ l) was added to alternethanoxin A (**24**, 2.0 mg) and dissolved in dry pyridine (40 μ l). The mixture was kept at room temperature. After 12 h, the reaction was complete, and MeOH was added. The pyridine was removed by a N₂ stream. The residue was purified by preparative TLC on silica gel [(eluent petrol-Me₂CO (7:3, v/v)] yielding the S-MTPA ester of alternethanoxin A, **28** (**Fig. 12**) as a homogeneous solid (R_f 0.39, 2.0 mg). It had: $[\alpha]_{25}^D$ - 12.7 (c 0.15); IR ν_{\max} 3374, 1771, 1725, 1637, 1595, 1284, 1214 cm⁻¹; UV λ_{\max} log (ϵ) 290 (3.9), 225 (sh) nm; ¹H NMR, δ : 7.97 (1H, d, J =7.5 Hz H-8), 7.51 (1H, dd, J =8.0 and 7.5 Hz, H-9), 7.46-7.31 (5H, m, Ph), 7.39 (1H, d, J =8.0 Hz, H-10), 6.37 (1H, s, H-6), 5.93

(1H, s, H-3), 3.76 (3H, s, OMe), 3.44 (3H, s, OMe), 2.23 (3H, s, MeCO); ESIMS (+) m/z 541 [M+Na]⁺.

4.1.6. (*R*)- α -Methoxy- α -trifluorophenylacetate (MTPA) triester of alternethanoxin A (29).

(*S*)-(+)-MPTA-Cl (20 μ l) was added to alternethanoxin A (**24**, 2.0 mg) and dissolved in dry pyridine (40 μ l). The reaction was carried out under the same conditions used for preparing **28** from **24**.

Purification of the crude residue by preparative TLC on silica gel [(eluent petrol-Me₂CO (7:3, v/v)] yielding R-MTPA ester of alternethanoxin A, **29** (**Fig. 12**) as homogeneous solid (R_f 0.55, 1.7 mg). It had: $[\alpha]_{25}^D$ -33.6 (c 0.13); IR ν_{\max} 1769, 1728, 1670, 1621, 1452, 1265, 1211, 1168 cm⁻¹; UV λ_{\max} (log ϵ) nm 287 (sh), 256 (4.62); ¹H NMR, δ : 87.58-7.25 (15H, m, Ph), 7.49 (1H, d, J =7.6 Hz, H-8), 7.19 (1H, dd, J =8.0 and 7.6 Hz, H-10), 6.92 (1H, d, J =8.0 Hz, H-10), 6.76 (1H, s, H-6), 6.72 (1H, s, H-3), 3.55 (3H, s, OMe), 3.52 (3H, s, OMe), 3.45 (3H, s, OMe), 3.35 (3H, s, OMe), 2.38 (3H, s, MeCO); ESIMS (+) m/z 973 [M+Na]⁺.

4.2. Biological assay

All the biological assays were carried out at the laboratory of All Russian Institute of Plant Protection, Pushkin (Saint Petersburg, Russia) under the supervision of Dr. A. Berestetskyi & Dr. G. Mitina whereas the zootoxic activity assay was carried out at the Saint Petersburg State Technical University under supervision of Dr. Vonokhodov.

4.2.1. Leaf-puncture assay.

Culture filtrates of *A. sonchi*, its organic extract, the chromatographic fractions and pure compounds **24-27** were assayed by leaf disc-puncture bioassay on *S. arvensis* and a number of non-host plants. The plants were produced from pieces of underground shoots or seeds and grown in a greenhouse. The discs (10 mm diam.) were cut off well-expanded leaves with cork borer, placed on moistened filter paper and punctured by sharp needle in the centre. Crude organic extract, chromatographic fractions and pure compounds were dissolved in a small amount of EtOH and then brought up to desirable concentration with distilled H₂O. The final concentration of EtOH in test solutions was 5% v/v that is non toxic to leaves of all plants in the control. Droplets (10 µl) of the test solution were applied on the discs and then incubated in transparent plastic boxes at 24°C under 12 h photoperiod. After 2 days of incubation the diameter of the necrotic lesions (mm) was measured.

4.2.2. Antimicrobial assay.

Antifungal activity of alternethanoxin A was assayed on *Saccharomyces cerevisiae*, *Candida tropicalis*, *Fusarium poa*, *Bipolaris sorokiniana*, *Rhynchosporium secalis*, *Penicillium* sp., *Aspergillus niger*, while alternethanoxins B was assayed only on *Saccharomyces cerevisiae*. Their antibacterial activity was tested on *Xanthomonas campestris*, *Escherichia coli* and *Bacillus subtilis* at the concentration 100 µg per disc according to the method previously described (Bottalico *et al.*, 1990).

4.2.3. Best solvent assay of alternethanoxin A.

Solubility of alternethanoxin A (**24**) in different solvents was tested in order to increase its activity. Solutions (2% and 5%) of methanol, ethanol, dimethyl sulfoxide, dimethylformamide, acetone, dioxane and acetonitrile were tested as control on leaves of *S. arvensis* and *C. arvensis* using leaf-puncture assay. All solvents used at both concentrations did not show phytotoxicity.

Alternethanoxin A, at the concentration 2 mg/ml, was dissolved in all solvents in solution at 5%, and applied on leaves of *S. arvensis* and *C. arvensis* using leaf-puncture assay, as described before.

4.2.4. Seeds germination assay.

Seeds of different plants were used to evaluate the effect of alternethanoxin A (**24**) on root growth. The seeds of lettuce, chicory, radish, wheat and pea were soaked for 5 minutes in a solution 1% of sodium hypochlorite, after washed with distilled water and incubated in water for 48 h at 25 °C. Seeds with rootlets of 1-2 mm length were incubated in a solution of DMFA at 5% together with the toxin at different concentrations (3.3×10^{-4} , 10^{-5} , 10^{-6} M) for 1 h at 25 °C. All seeds were replaced in transparent plastic boxes and incubated at 25 °C. The length of roots (mm) was measured at time 0 and after treatment. DMFA solution (5%) was used as a control treatment.

4.2.5. Zootoxic activity

The zootoxic activity was tested on *Paramecium caudatum*. Paramecia were grown in an infusion of 10 oat grains in 200 ml of water. 100 µl of a suspension with paramecia was added to 100 µl of a solution 5% DMFA of alternethanoxin A at different concentration (6.6×10^{-4} , 10^{-5} , 10^{-6} M). The solution was placed into a microscope slide and incubated in a humid chamber. After 3 min, 15 min, 1 h and 3 h of incubation, the cell integrity and activity of paramecia were estimated. A paramecium was considered dead if it became no motile and morphologically degraded (Biliai, 1982). The tested alternethanoxin A was considered to be severely toxic, toxic, and relatively toxic if no less than 70% of the paramecia died after 5, 20, and 40 min of incubation, respectively. If most of the paramecia remained morphologically unaltered after 60 min of incubation, the tested compound was considered non-toxic.

4.2.6. Electrolyte leakage assay

For the assay, leaf discs of *C. arvensis* (0.5 cm in diam.) treated with alternethanoxin A (2 mg/ml, 5 µl per disc) were cut for 4 peaces (10 discs per replication, 4 replications per treatment) and placed in 5 ml of distilled water. The discs treated with 5% DMFA were used for negative control. The discs boiled in water for several minutes were used as a positive control. The peaces of discs were incubated at 25 °C for 1 h and conductivity of resulted extract was measured by a conductivity meter (Mettler Toledo, Swirzerland, S20 SevenEasy).

4.2.7. Assay of inhibition of mitosis in onion roots.

The effect of alternethanoxin A on mitotic activity was tested using garlic (*Allium sativum* L.). Garlic cloves were allowed to grow in Petri dishes containing water in a growth chamber with constant fluorescent light at 25 °C. Cloves with 2-cm-long roots were selected and placed in a plastic dish containing 20 ml of alternethanoxin A solution of DMFA 5% at concentrations 3.3×10^{-4} , 10^{-5} , 10^{-6} M.

After 24 h, 3 mm of the root tip from germinated cloves were cut and fixed with Carnoy fixative (absolute ethanol:glacial acetic acid solution 3:1) for 5 minutes at room temperature, and for 1 h at -20 °C. The fixed root tips were stored in aceto-carmin for 2.0 minutes at room temperature. Root tips (2 mm) were sectioned using a dissection knife, mounted on slides in a drop of 45% acetic acid and examined under microscopy.

The remainder of the root was then discarded. Excess stain was blotted with a paper towel and the root tip was treated with a drop of deionised water. A coverslip was then lowered onto the root tip and firmly pressed in order to spread the cells into a single layer. The mitotic index, frequency of mitotic cells in prophase and frequency of interphases with multinucleolus were determinate. At least four root meristems from five different cloves were investigated at each fixation time. About 1000 cells per root were sampled to estimate the

mitotic index, frequency of mitotic cells in prophase and frequency of interphases with multinucleolus. Cloves with 2 cm roots treated with 5% of DMFA solution without alternethanoxin A served as control.

5. RESULTS AND DISCUSSION

5.1. Chemical characterization of alternethanoxins isolated from *A. sonchi* solid culture.

The solid culture of *A. sonchi* was exhaustively extracted as reported in the experimental section (Paragraph 4.1). The organic extract, showing a high phytotoxic activity on leaves of *S. arvensis*, was purified by a combination of column chromatography and preparative TLC on silica gel and reverse phase, as reported in the experimental section (Paragraph 4.1), giving two pure phytotoxic metabolites (**Scheme 1**).

Their close relationship was shown by ^1H and ^{13}C NMR investigations and they were named alternethanoxins A (**24**) and B (**25**) (**Fig. 9**) (51.0 and 2.2 mg/kg, respectively) on the basis of fungus source and their carbon skeleton.

Alternethanoxin A showed a molecular weight of 302 associated to a molecular formula $\text{C}_{16}\text{H}_{14}\text{O}_6$, consistent with ten unsaturations, nine of which were due to a 1,2,3-trisubstituted (A) and a pentasubstituted (C) aromatic rings and to a carbonyl group. In fact, the ^1H NMR (**Table 2**) and COSY (Berger and Braun, 2004) spectra (**Fig. 13** and **14** respectively) showed two doublets ($J=7.5$ and $J=7.1$ Hz) and a double doublet ($J=7.5$ and $J=7.1$ Hz) at the typical chemical shifts value for a suitable trisubstituted aromatic ring at δ 7.46 (H-8), 7.11 (H-10) and 7.34 (H-9) (Pretsch *et al.*, 2000). The same spectrum showed three singlets due to the proton (H-3) of the pentasubstituted aromatic ring and another proton, a methoxy and an acetyl groups at δ 6.21, 3.62 and 2.23 (Pretsch *et al.*, 2000). The singlet at δ 6.21, which integrated for two protons, was due to the overlapping of the H-3 signal and that of the proton (H-6) of the aldehyde group bonded at C-6 of the aromatic A ring and hemiacetalized with a phenolic group at C-5 of the aromatic C ring. These results were in full agreement with the absorption bands for hydroxy, conjugated carbonyl, and aromatic groups observed in the IR spectrum at 3341, 1697 and 1583 cm^{-1} (**Fig. 15**) (Nakanishi and Solomon 1977), as well as

with the absorptions maxima exhibited in the UV spectrum at 381, 299, and 241 nm (**Fig. 16**) (Pretsch *et al.*, 2000). These partial structures were supported by the data of the ^{13}C and DEPT spectra (**Fig. 17** and **18** respectively, **Table 2**) and the couplings observed in the HSQC spectrum (**Fig. 19**) (Berger and Braun, 2004). The aromatic protonated carbons, as well as, the methoxy and the acetyl groups were observed at the typical chemical shift value of δ 131.0, 122.2, 121.4, 109.5, 52.5 and 22.0 for C-9, C-8, C-10, C-3, MeO and MeCO, respectively (Breitmaier and Voelter, 1987). The same spectrum also showed the significant signals for the carbonyl and the hemiacetalic carbon (C-6) at δ 198.7 and 109.5, with the latter overlapped to the C-3 signal. The signals of the three and five quaternary carbons of the aromatic A and C rings resonated at very typical chemical shifts values of δ 167.3, 153.0 and 130.4 for C-7, C-6a and C-10a and 148.8, 160.0 (double signals), 128.6 and 109.8 for C-1, C-4 and C-5, C-2 and C-10b and were essentially assigned on the basis of the couplings observed in the HMBC spectrum (Berger and Braun, 2004) (**Fig. 20, Table 2**).

The couplings reported in **Table 2** also allowed to deduce the presence of a 2,6-pentasubstituted-2*H*-4-dehydropyran ring (B) accounting for the remaining unsaturation, which resulted joined to the other two rings (A and C) by the bridge-head carbons C-6a and C-10a and C-5 and C-10b, respectively. These findings allowed to assign the chemical shift to all the carbons and the corresponding protons (**Table 2**) as well as to alternethanoxin A the structure of a 1-(1,4,6-trihydroxy-7-methoxy-6*H*-benzo(d)chromen-2-yl)-ethanone (**24, Fig. 9**).

This structure was supported by other couplings observed in the HMBC spectrum (**Fig. 20, Table 2**) that showed, in particular, the correlation of C-1 and C-3 at δ 148.8 and 109.5 with the protons of methyl (δ 2.23) of acetyl group. Furthermore was observed the correlation between C-6a (δ 153) and the protons H-9 (δ 7.34) and H-10 (δ 7.11) of the aromatic ring A.

The C-7 (δ 167.3) correlated with the protons H-8 (δ 7.46) and the protons of methoxy group at δ 3.62.

The data from the HRESIMS spectrum (**Fig. 21**), recorded in positive modality, which showed sodium clusters formed by the toxin itself and the corresponding dimer at m/z 325.0701, $[M+Na]^+$ and 627 $[2M+Na]^+$ and the fragmentation peak at m/z 287 $[M-Me]^+$, which was generated by the molecular ion by loss of a methyl residue.

The structure of alternethanoxin A was confirmed by preparing two key derivatives whose spectroscopic properties were full consistent with the structure **24**. By usual acetylation with acetic anhydride and pyridine alternethanoxin A was converted into the corresponding triacetyl derivative **26** (**Fig. 10**), whose IR spectrum showed the significant absence of hydroxy groups and the presence of bands due to more ester carbonyl groups at 1770 and 1724 cm^{-1} (**Fig. 22**). Its ^1H and ^{13}C NMR spectra (**Fig. 23** and **24** respectively) differed from those of **24** for the significant presence of the signals of the three acetoxy groups at δ 2.02 and 1.95 (two MeCOO) and to δ 168.8 (two MeCOO), 168.5 (MeCOO) and 20.5 (three MeCOO). In the same spectra also the downfield shifts ($\Delta\delta=0.65$) of the overlapped signals of H-3 and H-6 at δ 6.86 and ($\Delta\delta=12.5$) of C-3 and C-6 at δ 122.0 were observed. The ESIMS spectrum (**Fig. 25**) showed sodium clusters formed by triacetylalternethanoxin A itself and the corresponding dimer at m/z 451, $[M+Na]^+$ and 879 $[2M+Na]^+$.

By reaction with an ethereal solution of diazomethane overnight at room temperature **24** was converted into the dimethyl ether derivative **27** (**Fig. 11**). Its ^1H NMR spectrum (**Fig. 26**) differed from that of **24** only for the presence of two more singlets due to the new methoxy groups at δ 3.74 and 3.31. Probably the phenolic hydroxy group at C-1 was not methylated as it was hydrogen bonded with the carbonyl group at C-2 generating a stable six-membered ring as showed by the singlet observed at typical chemical shift value of δ 12.95 (Pretsch *et al.*,

2000). The IR spectrum of **27** (Fig. 27) showed, in comparison with I spectrum of **24**, the absence of the hydroxy group band at 3341 cm^{-1} . Probably the proton of the hydroxyl group on C-1 formed an hydrogen bond with carbonil of acetyl group. The UV spectrum of **27** (Fig. 28) showed a maximum of absorbance at $\lambda_{\text{max}} 285\text{ nm}$.

The ESIMS spectrum (Fig. 29) of **27** showed sodium clusters formed by dimethyl alternethanoxin A itself and the corresponding dimer at m/z 353 $[\text{M}+\text{Na}]^+$ and 683 $[2\text{M}+\text{Na}]^+$.

Alternethanoxin B showed a molecular weight of 300 associated to a molecular formula of $\text{C}_{16}\text{H}_{12}\text{O}_6$ as deduced from its HRESIMS spectrum and consistent with eleven unsaturations. It differs from alternethanoxin A for the lacking of two hydrogens and one unsaturation more. They showed very similar IR and UV spectra (Fig. 30 and 31 respectively), while the comparison of their ^1H , ^{13}C and DEPT spectra (Fig. 32, 33 and 34, Table 3) showed a very close structures with the only difference in the substitution of the aromatic A ring. In fact, its ^1H NMR spectrum showed two *ortho*-coupled aromatic protons resonating as doublets ($J=9.0\text{ Hz}$) at δ 7.47 and 7.36 and assigned to H-9 and H-8, which coupled in the HSQC spectrum (Fig. 35) with the aromatic protonated carbons at δ 122.2 and 125.4, and the absence of H-10 (Pretsch *et al.*, 2000, Breitmaier and Voelter, 1987). The ^{13}C NMR spectrum also showed the significant downfield shift ($\Delta\delta=30.8$) of C-10 attributable to the presence of a tetrasubstituted furan ring, accounting for the additional unsaturation. This new ring probably was generated by the attachment of the oxygen at C-1 of C ring to the carbon C-10 of A ring.

The COSY spectrum (Fig. 36) showed the absence of H-10, while the signals of H-8 and H-9 (δ 7.36 and 7.74 respectively) appeared as two doublets ($J= 9.0\text{ Hz}$) at δ 7.36 and 7.74. This is characteristic coupling for two *ortho* proton, a tetra substituted aromatic ring. This partial structure was also consistent with the couplings observed in HSQC spectrum (Fig. 35), that also showed a further quaternary oxygenated carbon C-10. The assigned of the

quaternary carbons was made on the basis of couplings observed in the HMBC spectrum (**Fig. 37, Table 3**).

Furthermore, the examination of the ^1H NMR and COSY spectra also showed a different chemical shift values for the protons (H-3) of the pentasubstituted aromatic C ring and that of the hemiacetalized aldehyde group (H-6) resonating at δ 6.73 and 6.62, respectively, which coupled in the HSQC with the signals at δ 107.2 and 111.4 (C-3 and C-6), respectively. These findings suggested an opposite stereochemistry at C-6 in **25** in respect of **24**, which was also supported by the presence in the ^1H NMR spectrum of **25** of a singlet at δ 12.20 due to the hemiacetalic hydroxy group, which is probably hydrogen bonded to the methoxy group at C-7 and generating a stable six-membered cycle. This result was confirmed by the couplings observed in the NOESY spectra (Berger and Braun, 2004) of **24** and **25**. In fact, the NOESY spectrum of **24**, beside the expected effect observed between H-3 and the methyl of the acetyl group at C-2, also showed an effect between H-6 and the methoxy group at C-7. This latter effect was significantly absent in NOESY spectrum of **25**.

These findings allowed to assign the chemical shift values to all the carbons and the corresponding protons (**Table 3**) and to alternethanoxin B the structure of 1-(7,9-dihydroxy-1-methoxy-9H-4,8-dioxacyclopenta[def]phenanthren-5-yl)-ethanone (**25, Fig. 9**). This structure was supported by the other couplings observed in the HMBC spectrum (**Table 3**) and by the data of the HRESIMS (**Fig. 38**), recorded in positive modality, which showed sodium clusters formed by the toxin itself and the corresponding dimer at m/z 323.0541, $[\text{M}+\text{Na}]^+$ and 623 $[\text{2M}+\text{Na}]^+$.

The absolute stereochemistry of the secondary hydroxylated carbon C-6 of alternethanoxin A (**24**) was determined applying the Mosher's method (Dale et al., 1969; Ohtani et al., 1991). By reaction with the *R*-(-)- α -methoxy- α -trifluorophenylacetate (MTPA) and *S*-(+)MTPA chlorides, alternethanoxin A was converted in the corresponding

diastereomeric *S*-MTPA ester and *R*-MTPA triesters (**28** and **29**, **Fig. 12**), whose spectroscopic data were consistent with the structure assigned to **24**. In particular, the IR spectrum of **28** (**Fig. 39**) showed the presence of an hydroxy group band at 3374 cm^{-1} and the presence of a band at 1771 cm^{-1} for the presence of the ester carbonyl group. The UV spectrum (**Fig. 40**) showed a maximum of absorbance at λ_{max} 290 nm. The ESIMS spectrum (**Fig. 41**) recorded in positive modality showed the presence of sodium cluster at m/z 541 $[\text{M}+\text{Na}]^+$.

The IR spectrum of **29** (**Fig. 42**) showed, in comparison with the spectrum of **24**, the absence of the hydroxy group band, and the presence of bands due to more ester carboxylic group at 1769 and 1728 cm^{-1} . The UV spectrum (**Fig. 43**) showed a maximum of absorbance at λ_{max} 256 nm. The ESIMS spectrum (**Fig. 44**) recorded in positive modality showed the presence of sodium cluster at m/z 973 $[\text{M}+\text{Na}]^+$.

The comparison between the ^1H NMR data of the *S*-MTPA ester (**28**, **Fig. 45**) and those of the *R*-MTPA triester (**29**, **Fig. 46**) of **24** [$\Delta\delta$ (**28-29**): H-3 -0.79; H-8 +0.48; H-9 +0.42; H-10 +0.49 MeO +0.21 and MeCO -0.15] allowed to assign a *R*-configuration at C-6. In alternethanoxin B C-6 has an opposite stereochemistry in respect to **24**, so that a *S*-configuration could be assigned to this chiral carbon in **25**.

5.2. Biological activity of alternethanoxins.

Compounds **24-27** were tested by leaf disc-puncture assay at a range of concentrations from 0.1 to 4 mg/ml on leaf discs of *S. arvensis*.

Only alternethanoxins A and B (**24** and **25**) were shown to be phytotoxic. Small necrotic lesions were seen at the concentration of 0.12, 0.25, 0.5, 1 and 2 mg/ml for **24** and **25**, respectively. At the highest concentration of **24** and **25** (4 mg/ml) lesions reached 3 mm in diameter, respectively (**Fig. 47a** and **47b**, **Fig. 48a** and **48b**).

When tested at the concentration 2 mg/ml on leaf discs of a number plant species (*Sonchus arvensis*, *Cirsium arvense*, *Taraxacum officinalis*, *Aegopodium podagaria*, *Trifolium pratense*, *Phelum pratense*, *Rumex obtusifolia*, *Chenopodium album*, *Cannabis sativa* and *Elytrigia repens*) alternethanoxins A and B showed similar non-specific activity (lesions ~ 1-2 mm diameter; **Fig. 49a** and **49b**).

Furthermore, the inactivity of derivatives **26** and **27** demonstrated that the phenolic hydroxy group at C-4 of C ring is a structural feature important for the phytotoxicity while the activity of alternethanoxin B showed that the other one at C-1 and the hemiacetal hydroxyl group at C-6 are unessential. The reduction of both the hemiacetal and the acetyl groups at C-6 and C-2 and the electrophilic substitution of one or more hydrogens of ring A and C rings with a suitable group could contribute to demonstrate the importance of the benzo(d)chromene moiety and the role of the acetyl group. Both **24** and **25** demonstrated neither antibiotic nor antifungal activity when tested at 100 µg/disc on *Bacillus subtilis*, *Xanthomonas campestris*, *Escherichia coli* and *Saccharomyces cerevisiae*.

Alternethanoxins A and B are two fungal metabolites in which an ethanone group was bonded to an original polysubstituted benzo(d)chromene and dioxacyclopenta[def]-phenanthrene residue, respectively, and occur for the first time as natural compounds with

interesting biological activity. In particular, the main fungal metabolite alternethanoxin A (**24**) and also alternethanoxin B (**25**) showed potential herbicidal properties.

A number of well-known fungal metabolites (alternariol, its monomethyl ether, altenuene, and altenuisol), which belong to a class of toxic metabolites containing dibenzo[α]pyrone moiety, are structurally close to alternethanoxin A. These compounds are produced by different *Alternaria* species isolated from plant material and their antibiotic, cytotoxic and teratogenic activities are usually stressed (Cole and Cox, 1981). Interestingly, that alternethanoxins A and B did not demonstrated antimicrobial activity. Furthermore, the most closest compounds to alternethanoxin A from the group of ethanones appeared to be the acetophenones, namely cynandiones A-D, cynanhone and analogues, isolated from the root of different *Cynanchum* plant species and showing potential pharmacological applications (Huang *et al.*, 1999). Compounds close to alternethanoxin B are those belong to the cyclopenta[d,e,f]phenathrene group including the steriols, toxic metabolites produced by some *Fusarium sporotrichiella* strains, isolated from naturally infected grain (Olifson *et al.*, 1961).

Taking in the consideration the structural relation of alternethanoxins A and B to some mycotoxins of *Alternaria* spp., it was interesting to assay activity on more species of microorganisms. Therefore, alternethanoxins A was tested against fungi *Candida tropicalis*, *Fusarium poa*, *Bipolaris sorokiniana*, *Rhynchosporium secalis*, *Penicillium* sp., *Aspergillus niger*, and this assays demonstrated that alternethanoxin A did not showed antifungal activity at concentration 100 $\mu\text{g}/\text{disc}$.

The toxicity of alternethanoxin A and B was tested on leaves of *S. arvensis* in combined application. The toxins are present in fungal culture in mixture, so it was interesting to test their synergistic effect. Antagonist effect was found in action of these compounds, possibly they interact with the same molecular target and interfere action of each other. The necrosis

evaluated on leaves of host plant, showed less diameter when the two toxins were applied together, instead of both toxins applied alone (**Fig. 50**).

Considering the enough amount of alternethanoxin A available, other assays were conducted for the biological investigation of this toxin.

The best solvent for the solubilization of alternethanoxin A was determinate to be dimethylformamide.

5.2.1 Effect of concentration of alternethanoxin A on root growth.

The ability of alternethanoxin A to inhibit root growth in seeds of different plants (lettuce, chicory, radish, wheat and pea) was investigated. All seedling were sensitive and the toxin inhibited significantly the root growth of seeds of lettuce (88% at 1.6×10^{-4} M). Wheat was find less significantly sensitive plant (68% at 1.6×10^{-4} M) then lettuce, while sensitivity of chicory, radish and pea was intermediate (**Fig. 51** and **52**). This results show that alternethanoxin A is is able to inhibit root growth non-selectively.

5.2.2. Effect of alternethanoxin A on zootoxic activity.

Paramecium caudatum test model (Protozoa subkingdom) is widely used to study the biologic activity of various drugs (Green *et al.*, 1989). *P. caudatum* has morphological signs of a cell and responds to environmental stimuli similarly to multicellular organisms.

Alternethanoxin A did not show zootoxic activity when tested on the infusorium *Paramecium c.* Even after 3 h of treatment with the toxin 100% of infusoria remained viable.

5.2.3. Effect of alternethanoxin A on conductometric properties of *C. arvense* leaves.

The effect of Alternethanoxin A on electrolyte leakage in leaves of *Cirsium arvense* was investigated. Leaf discs treated with alternethanoxin A in the light showed an increment of conductivity significantly different in comparison with discs treated with the toxin in the dark. Both the positive controls (light and dark) show an increment of conductivity in comparison with the negative control (100%). This results showed that the action of alternethanoxin A is light-dependent (**Fig. 53**). This toxin could be involved in disruption of some photosynthesis process. Lost of membrane integrity occurring only in tissues exposed to light may be associated with compounds that act as photosynthetic electron diverters (i.e., bipyridiliums) or cause photodynamic pigment to accumulate (i.e., inhibitors of protoporphyrinogen oxidase).

5.2.4. Effect of alternethanoxin A on inhibition of mitosis in onion roots.

The differences in mitotic index in onion roots cell's were measured after treatment with alternethanoxin A. The mitotic index was obtained by dividing the total number of cells undergoing mitosis by the total number of cells observed (**Table 4**). At least three replicates should be included for each treatment with a minimum of 1000 cells, and their various states in mitosis are recorded per replication. In addition to important quantitative data, visual observation of the root squashes also may detect an abnormal mitotic arrangement or atypical cell wall formation that would suggest either a disruption of the microtubule-organizing centers or alteration of processes involved in cell wall biosynthesis. Considering the % of cells presented in different phases of mitosis (prophase, metaphase, anaphase, telphase) in comparison with the total cells taking in examination, as expressed in **Table 4**, after treatment with the toxin a decrease of cells in metaphase and telophase was observed. The

most cells examined show an abnormal metaphase, with several swollen cells, bigger than normal, and some dead cells. Also several cells showed the presence of a vacuole inside the nucleolus that could indicate the beginning of the cell's death. More cells in prophase means the inhibition of the division processes. More cells in telophase means appearance binucleate cells without septa and division (**Fig 54**). It means that alternethanoxin A is a potent inhibitor of microtubule assembly.

6. CONCLUSION

1. The fungus *Alternaria sonchi* was selected as a pathogen of *Sonchus arvensis*.
2. The best conditions for the production in solid culture of phytotoxic metabolites were found.
3. Bioassay-guided purification of the organic extract of the solid culture allowed to isolate two new metabolites with phytotoxic activity.
4. Two phytotoxic metabolites named alternethanoxins A and B, were characterized by extensive use of spectroscopic (essentially NMR and MS techniques) and chemical methods, as new phytotoxic polycyclic ethanones.
5. Alternethanoxins A and B showed a significant phytotoxic activity against host plant and other several weeds.
6. Structure-activity relationship studies testing phytotoxic activity, showed that the hydroxy group at C-4 is an important factor to impart activity while the other two on C-1 and C-6 appear to be unessential.
7. Alternethanoxins A and B didn't showed antimicrobial activity.
8. Application of both alternethanoxins on leaves of host plant did not showed synergistic effect.
9. Other experiments were carried out with alternethanoxin A:
 - a) The best solvent for the toxin appeared dimethylformamide.
 - b) The toxin inhibited root growth non-selectively.
 - c) The toxin did not showed zootoxic activity.
 - d) The toxin increased conductivity on leaves of *C. arvensis*, and this activity was light-dependent.
 - e) The toxin was found to be a potent inhibitor of mitosis process in onion roots.

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Fig. 1. *Cirsium arvense*



Fig. 2. *Sonchus arvensis*

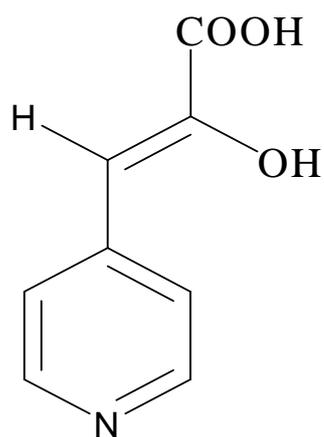
**1**

Fig. 3. Structure of ascosonchine (**1**) isolated from *Ascochyta sonchi* culture filtrates.

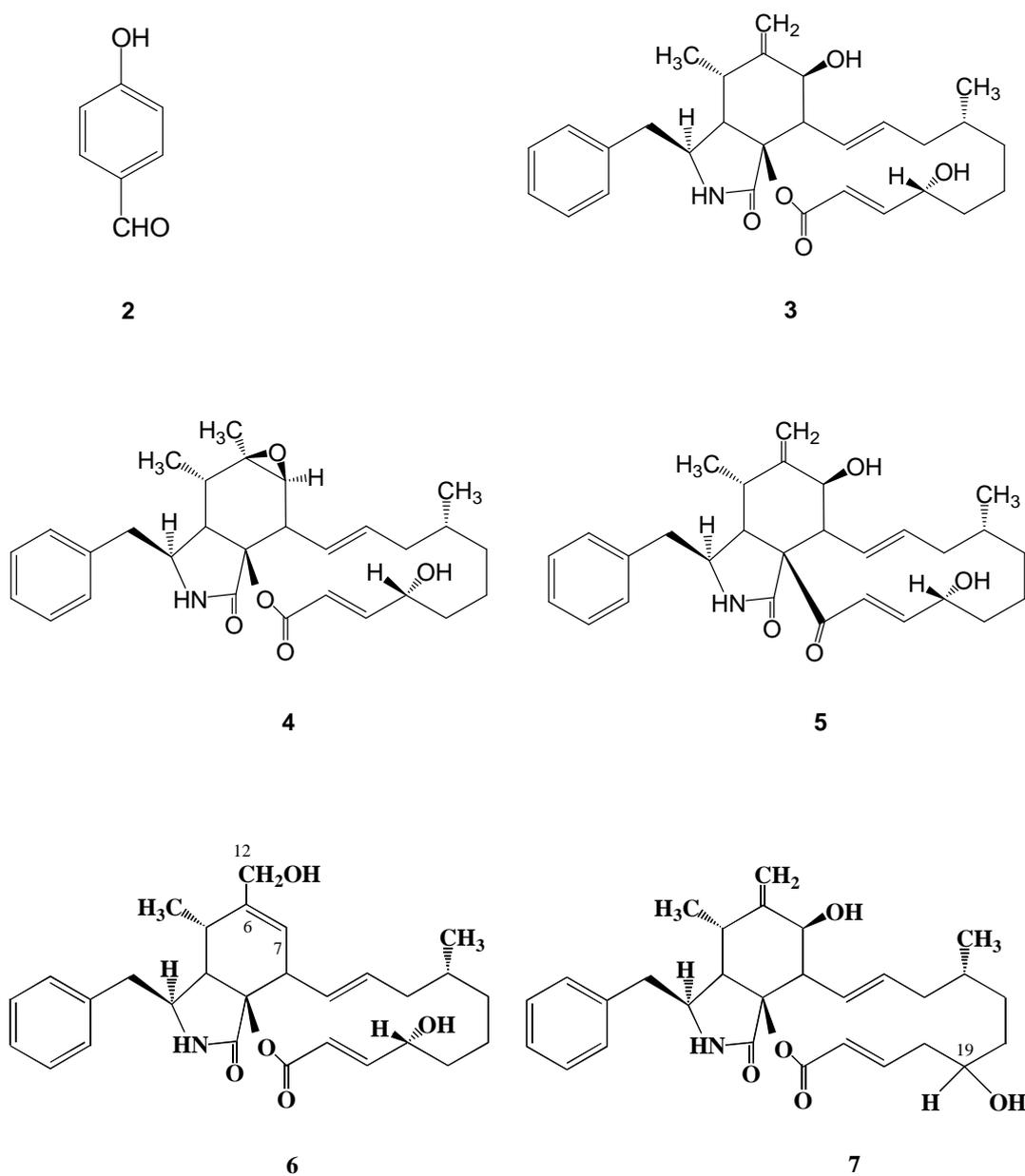
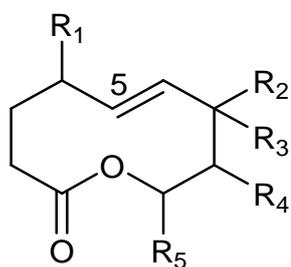


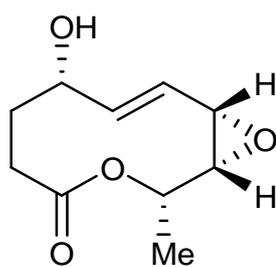
Fig. 4. Structure of *p*-hydroxybenzaldehyde (**2**), cytochalasins B and F, and deoxaphomin (**3**, **4** and **5**) isolated from liquid and solid cultures of *P. exigua* var. *exigua* strain C-177; structure of cytochalasins Z2 and Z3 (**6** and **7**) isolated from a solid culture of *P. exigua* var. *exigua* strain S-9.



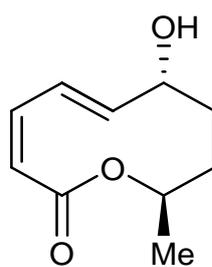
8 $R_1=H$, $R_2+R_3=O$, $R_4=\alpha-OH$, $R_5=\beta-CH_2CH_2CH_3$

9 $R_1=\beta-OH$, $R_2=\beta-H$, $R_3=\alpha-OH$, $R_4=\alpha-OH$, $R_5=\beta-CH_2CH_2CH_3$

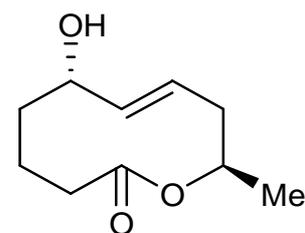
10 $R_1=\alpha-OH$, $R_2=\alpha-H$, $R_3=\beta-OH$, $R_4=H$, $R_5=\beta-Me$



11



12



13

Fig. 5. Structure of stagonolide A (**8**), isolated from liquid culture of *S. cirsi*, and stagonolides B-F (**9-13**), isolated from the same fungus, in solid culture.

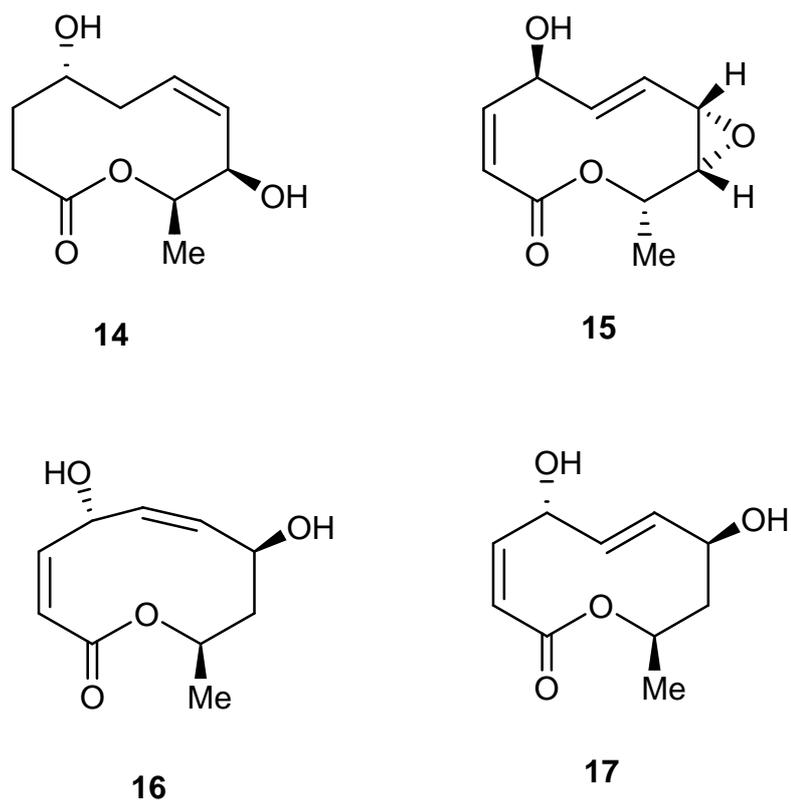


Fig. 6. Structure of stagonolides G-I (14-16) and modiolide A (17), isolated from liquid culture of *S. cirsii*.

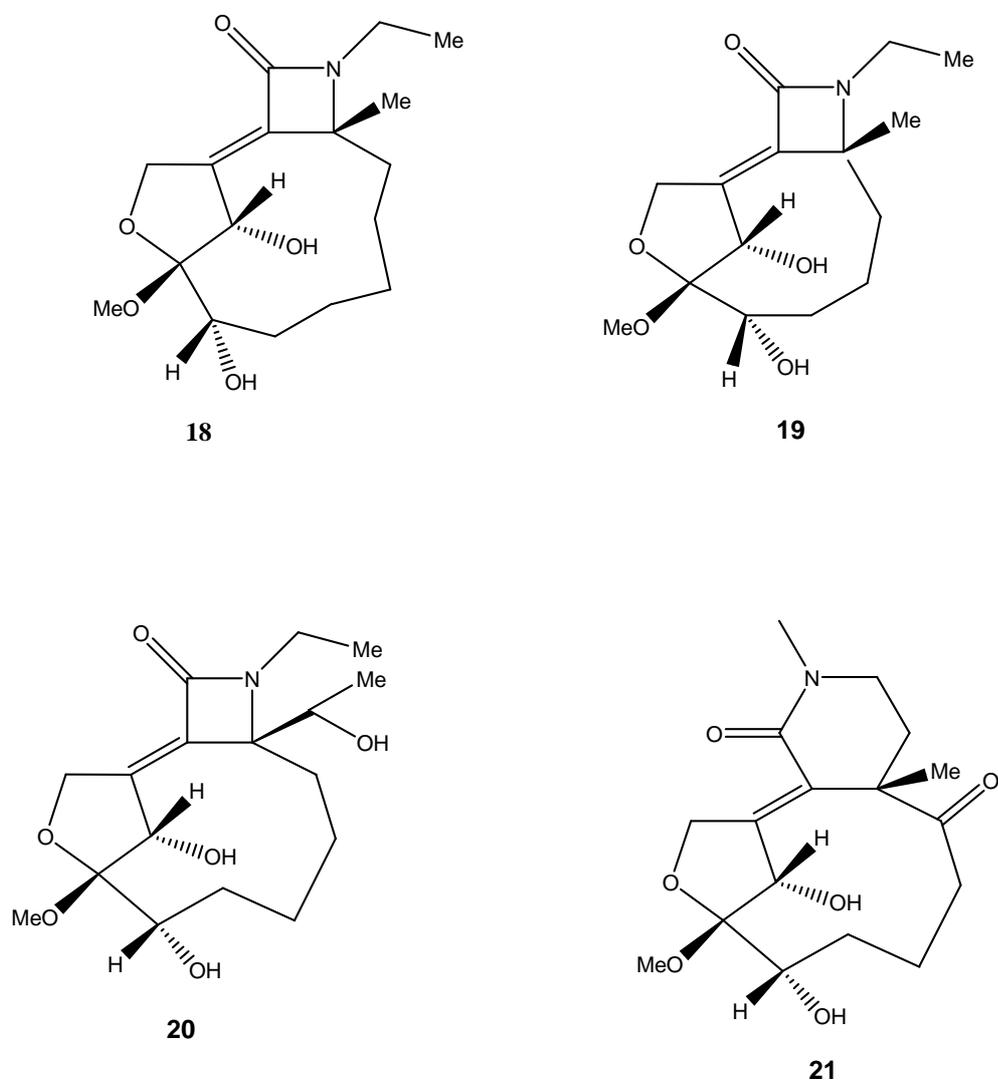


Fig. 7. Structure of phyllostictines A-D (**18-21**) isolated from liquid culture of *P. cirsii*.

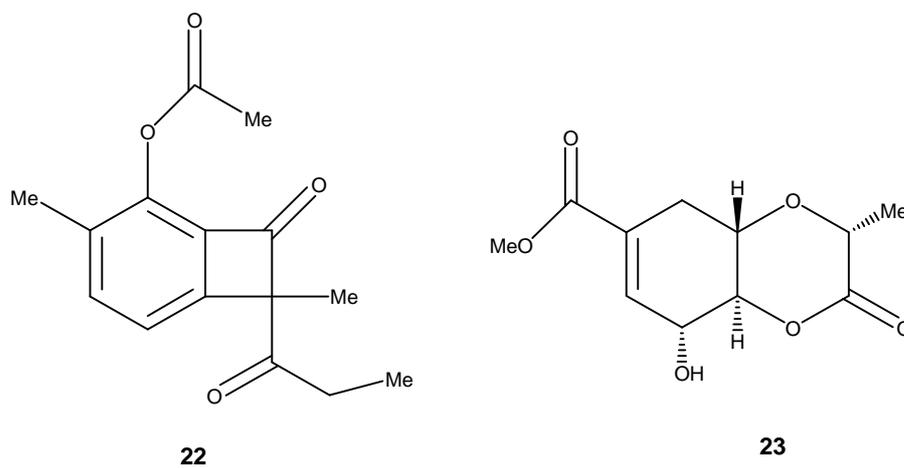


Fig. 8. Structure of phyllostoxin and phyllostin (1 and 2) isolated from liquid culture of *P. cirsi*.

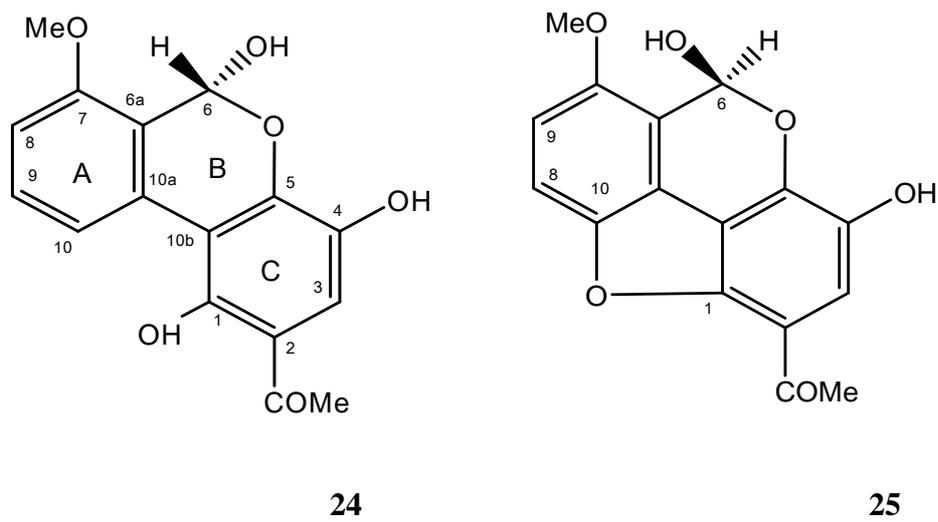
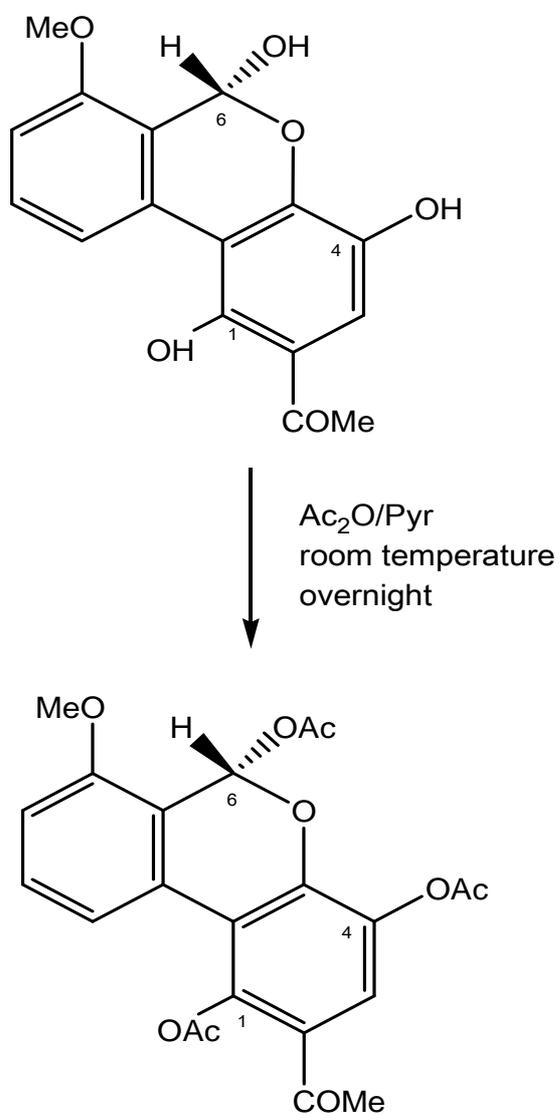
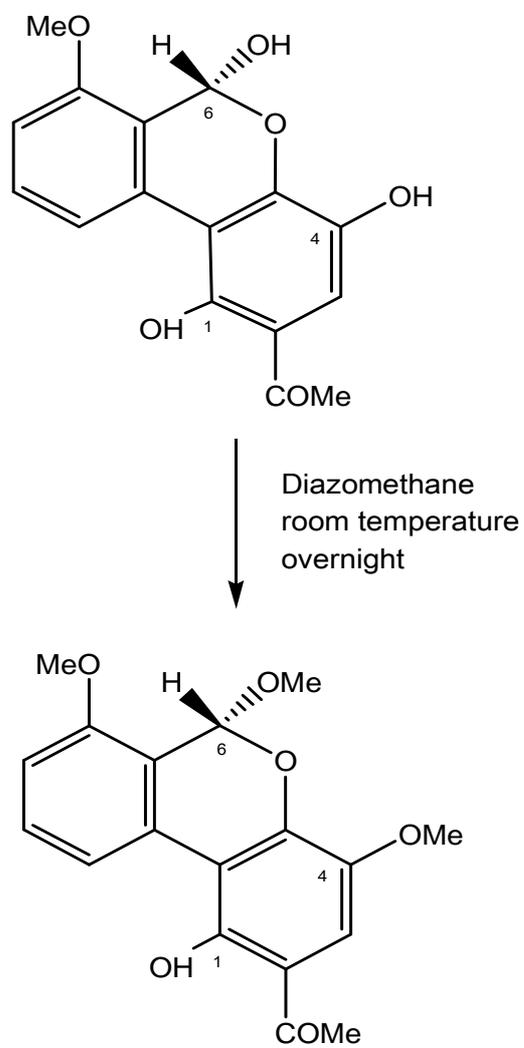


Fig. 9. Structure of alternethanoxins A and B (**24** and **25**) isolated from *Alternaria sonchi*.



26

Fig. 10. Acetylation of alternethanoxin A.



27

Fig. 11. Methylation of alternethanoxin A.

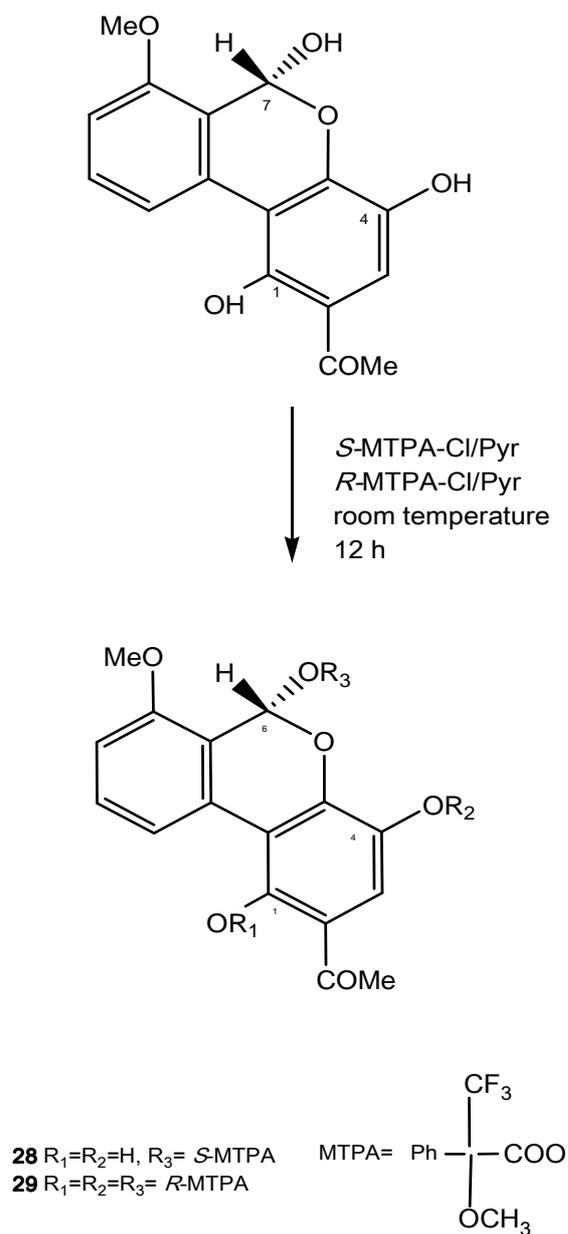


Fig. 12. Preparation of *S*-(-)- and *R*-(+)- α -methoxy- α -trifluorophenylacetate (MPTA) of alternethanoxin A (**28** and **29**).

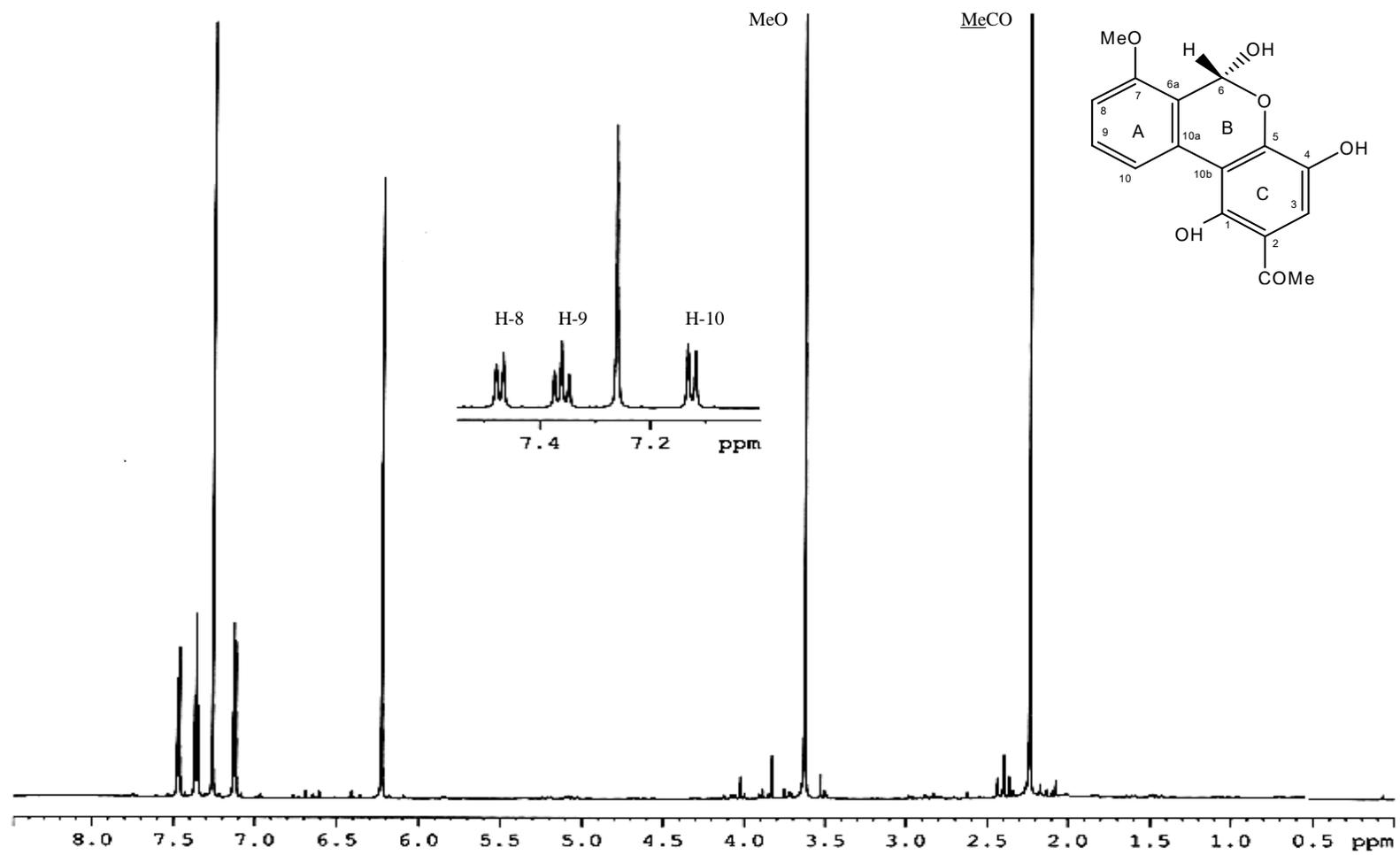


Fig. 13. ^1H NMR spectrum of alternethanoxin A recorded at 600 MHz.

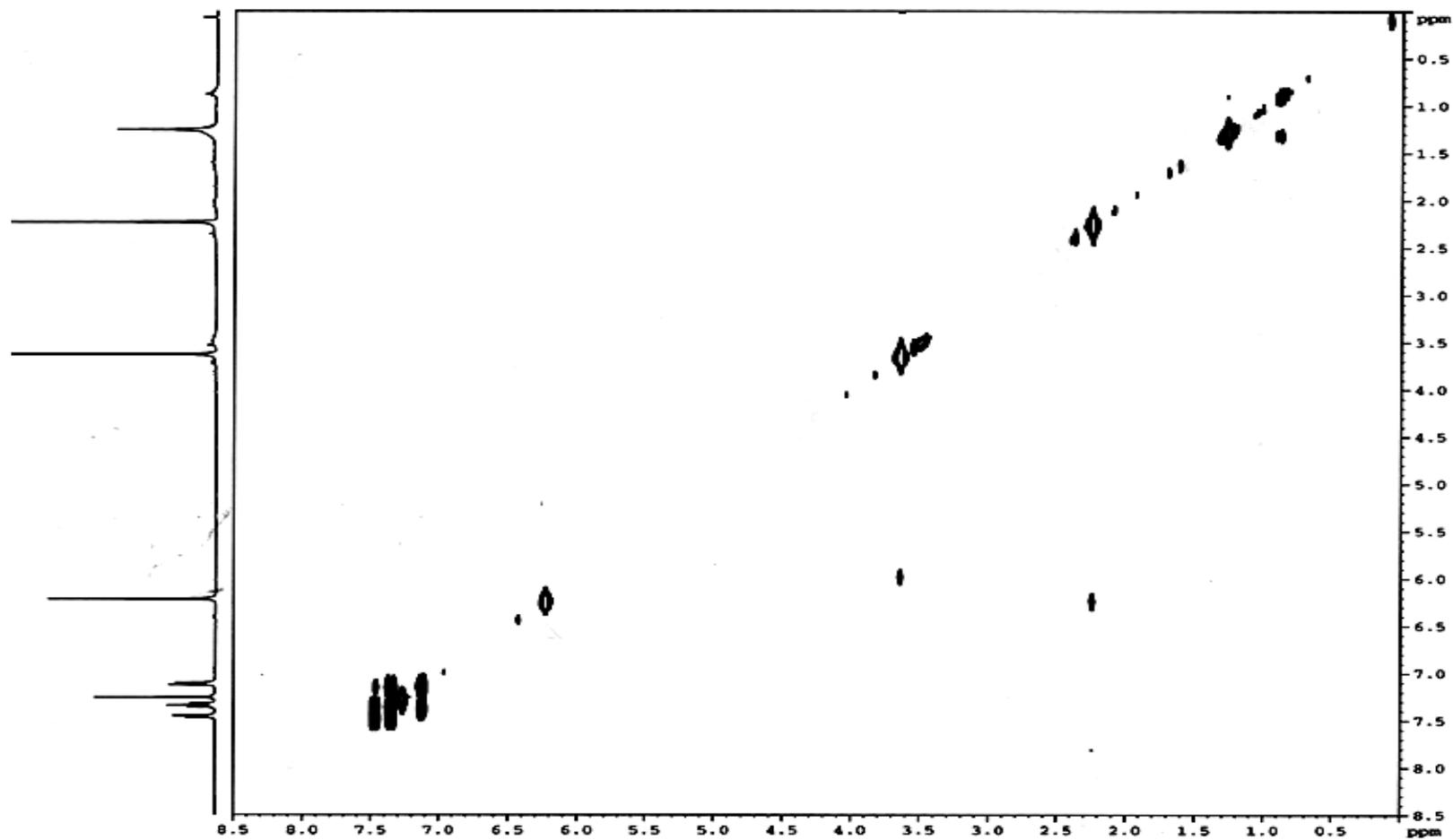


Fig. 14. COSY spectrum of alternethanoxin A recorded at 600 MHz.

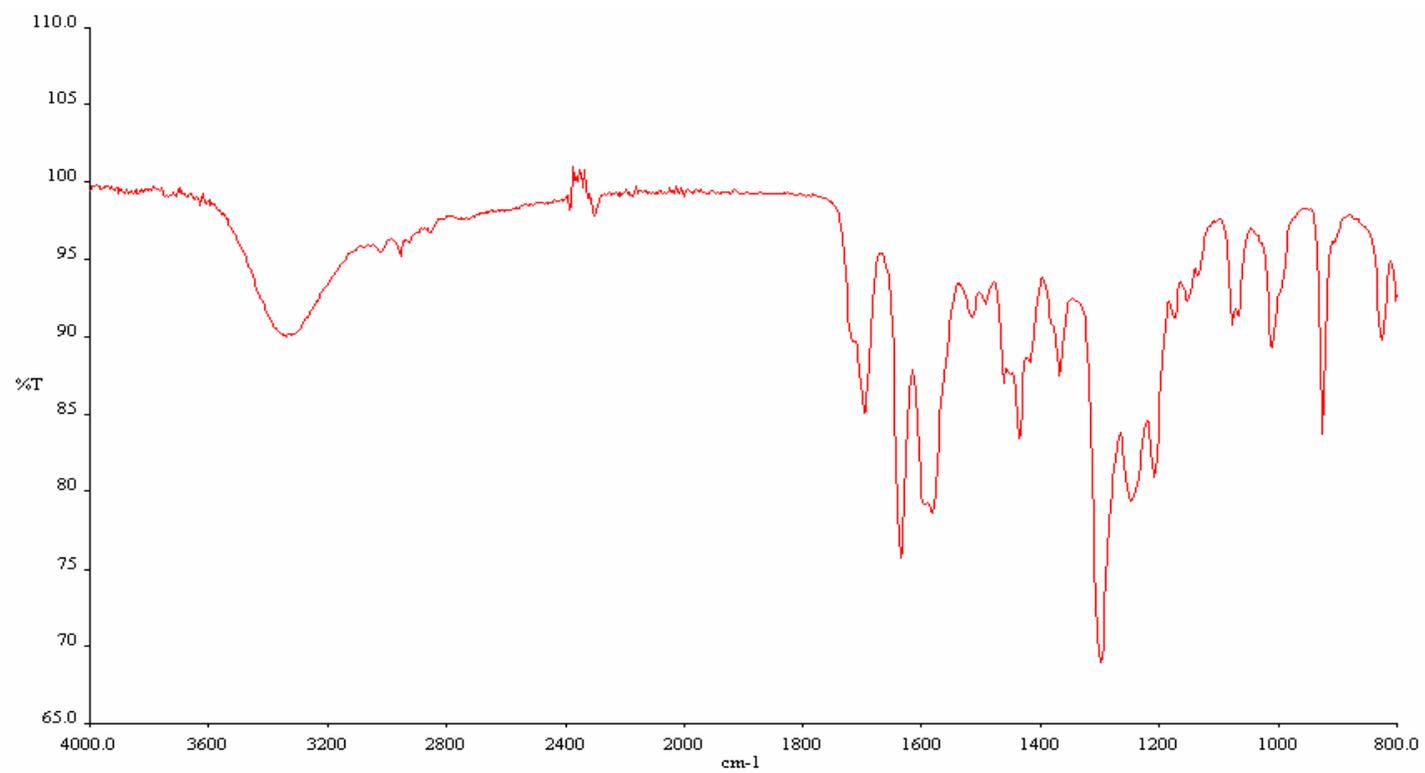


Fig. 15. IR spectrum of alternethanoxin A deposited glassy film.

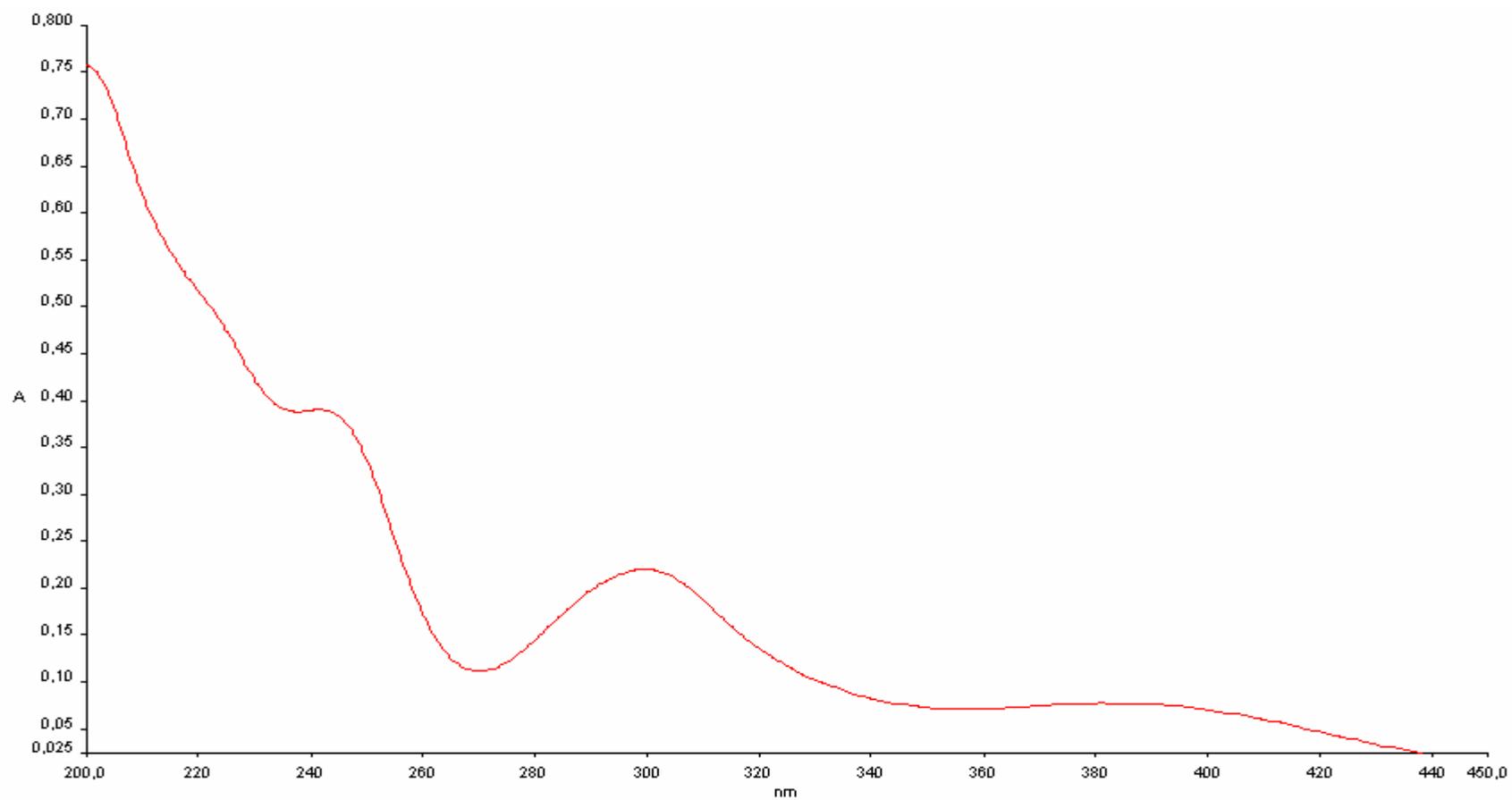


Fig. 16. UV spectrum of alternethanoxin A recorded in MeCN solution.

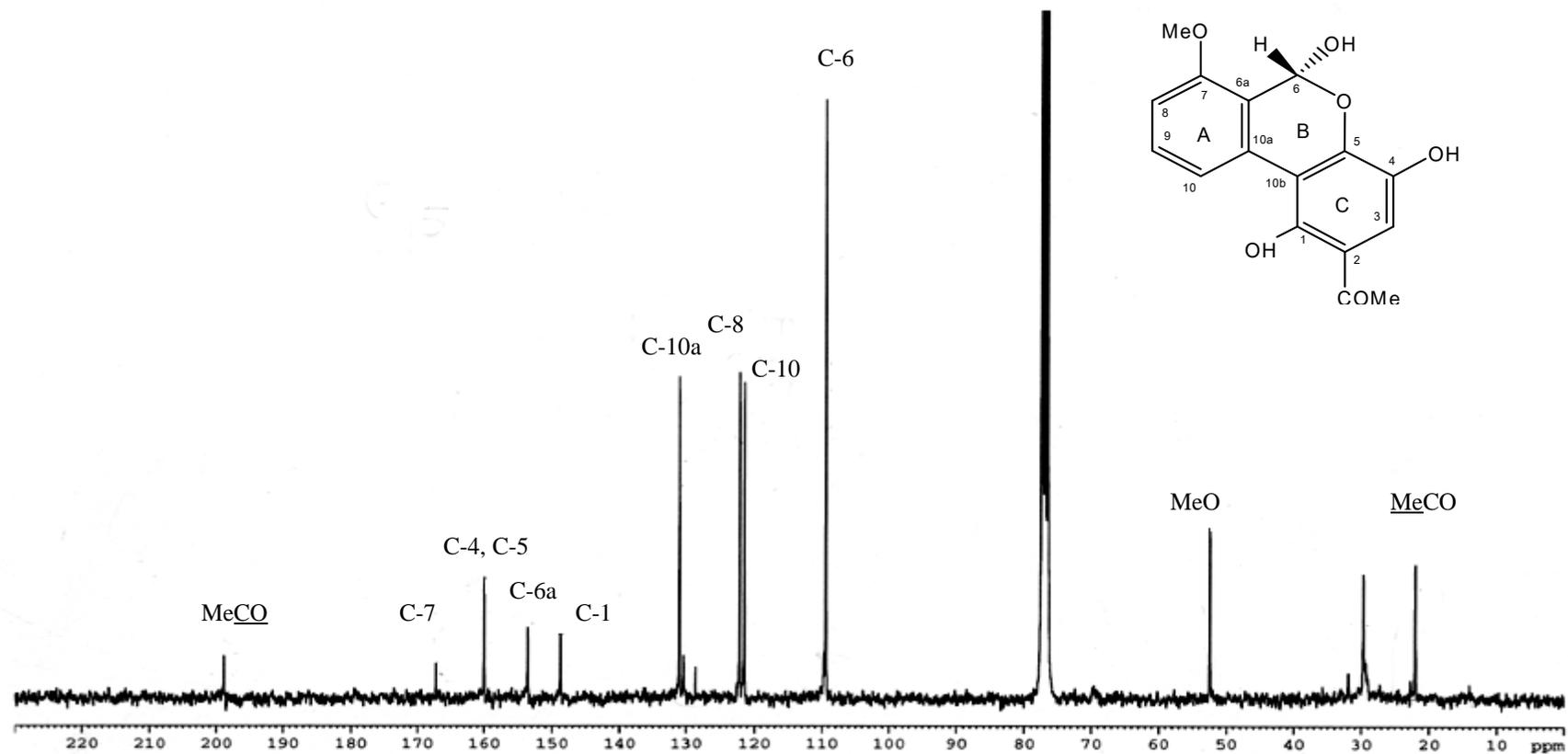


Fig. 17. ^{13}C NMR spectrum of alternethanoxin A recorded at 600 MHz.

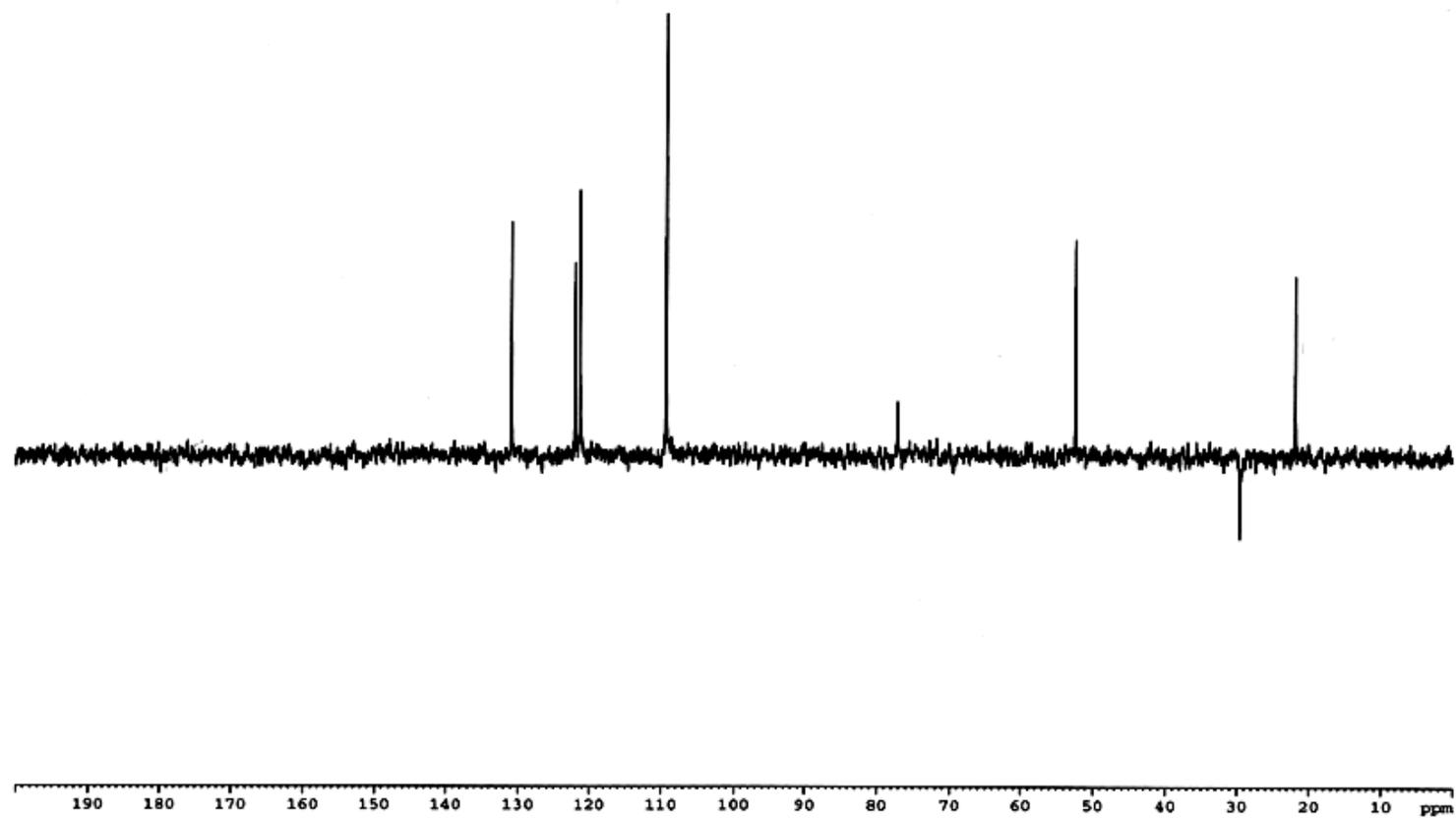


Fig. 18. DEPT spectrum of alternethanoxin A recorded at 600 MHz.

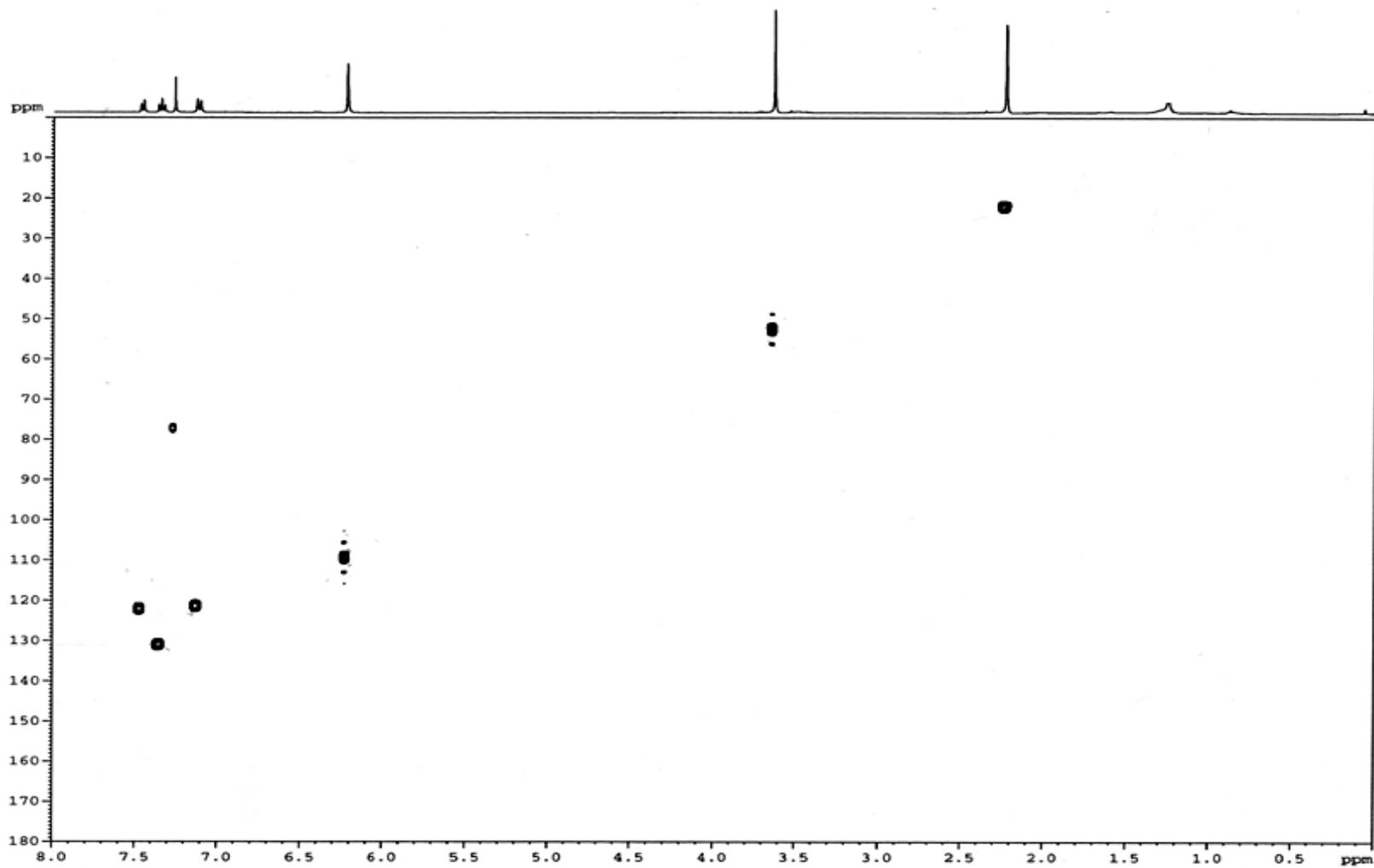


Fig. 19. HSQC spectrum of alternethanoxin A recorded at 600 MHz.

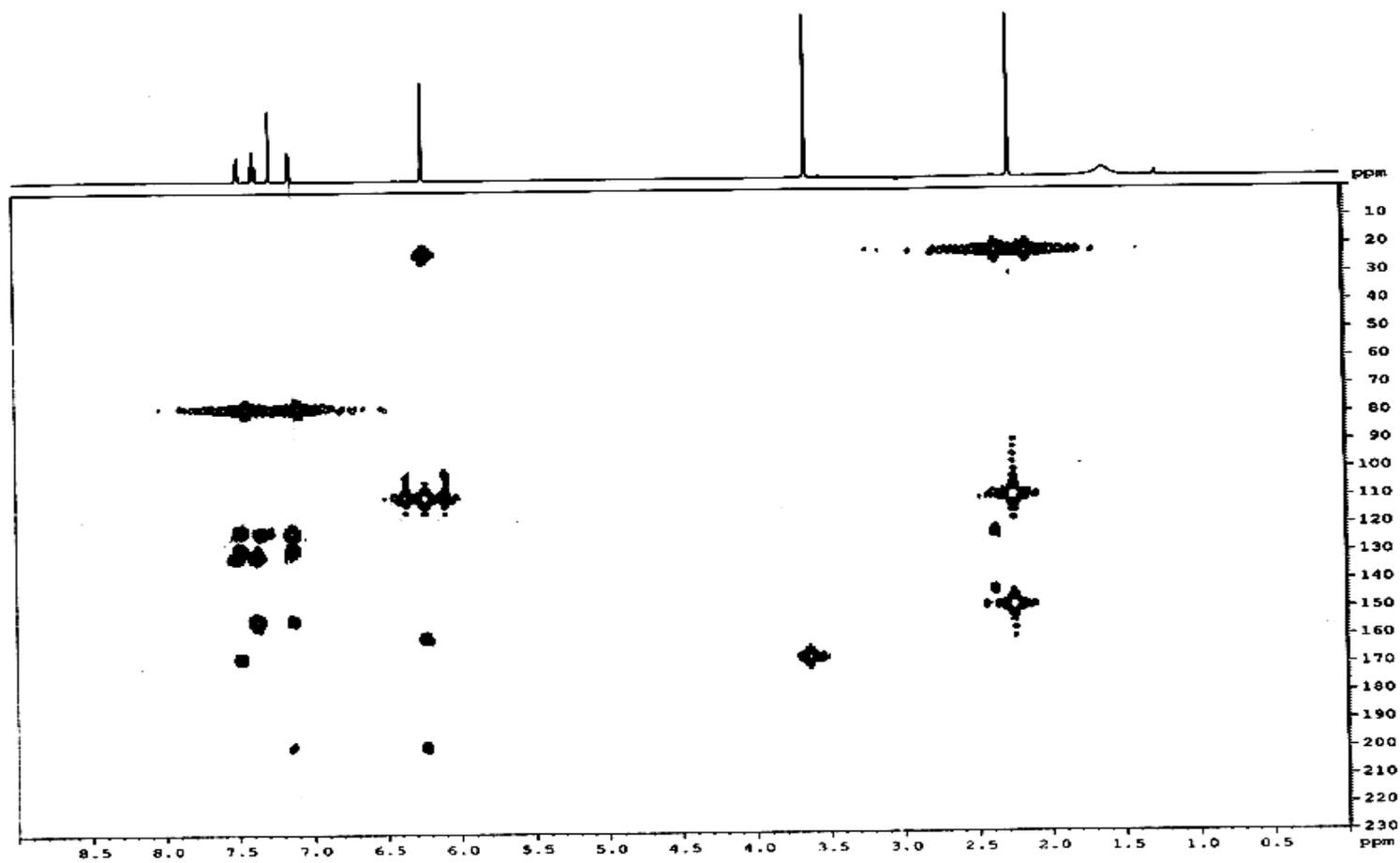


Fig. 20. HMBC spectrum of alternethanoxin A recorded at 600 MHz.

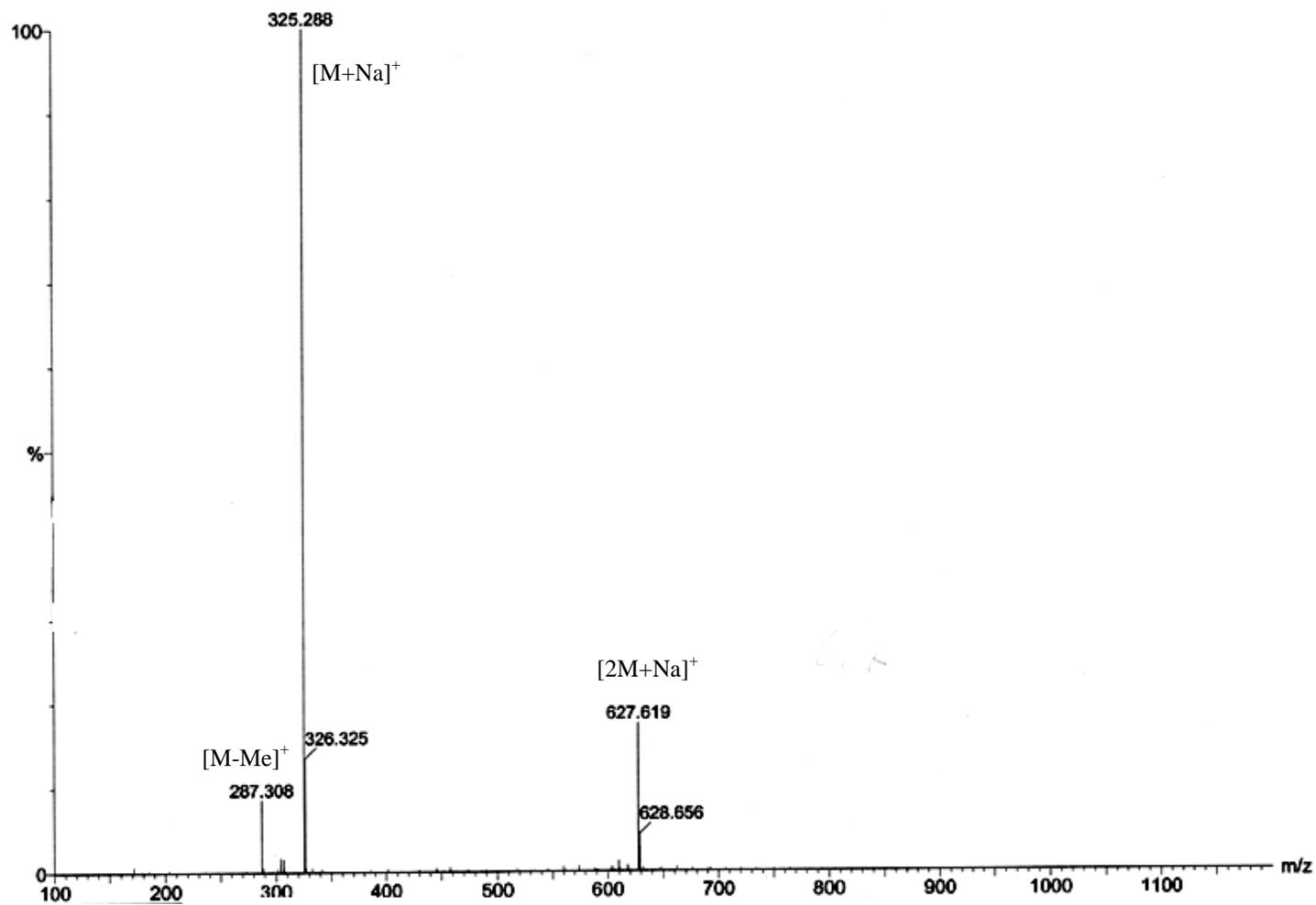


Fig. 21. ESI MS spectrum of alternethanoxin A recorded in positive modality.

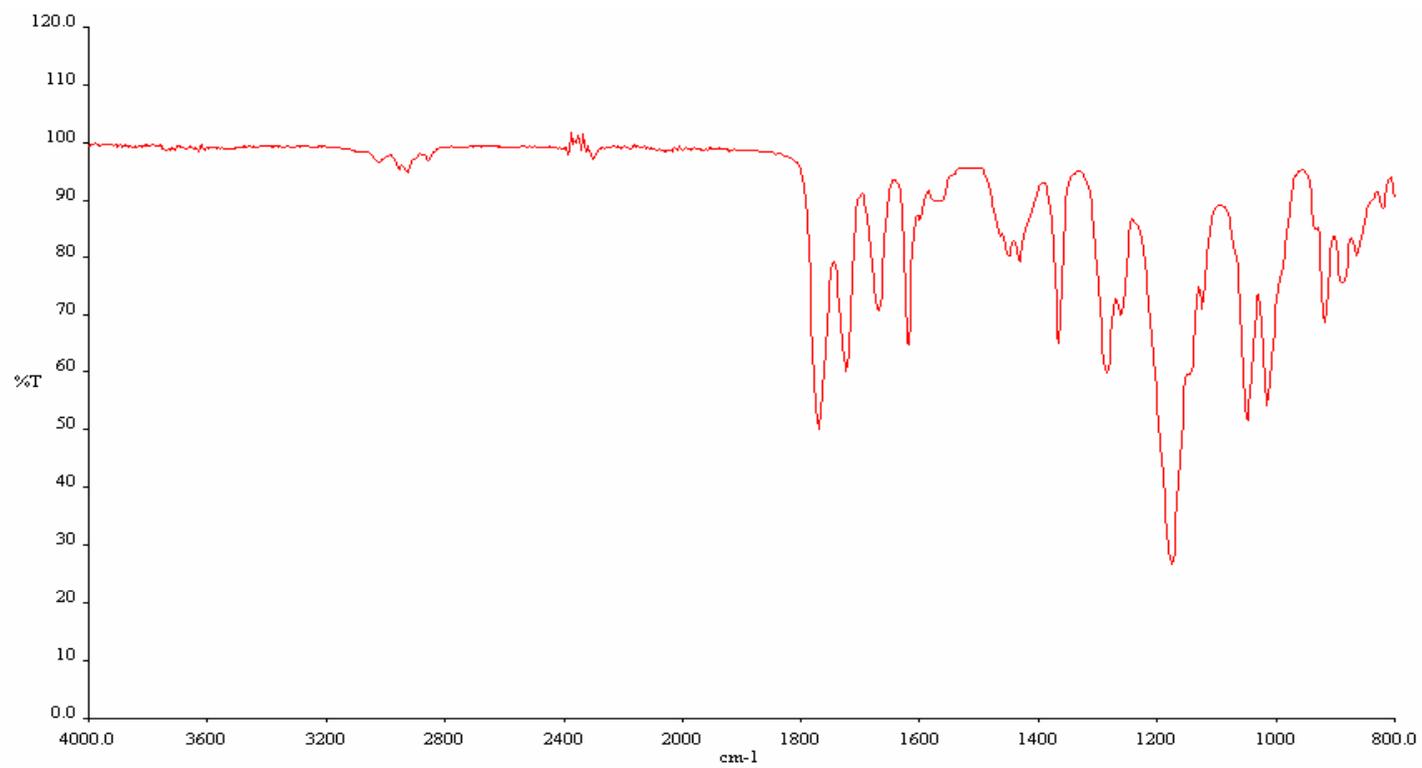


Fig. 22. IR spectrum of triacetylalthernethanoxin A deposited glassy film.

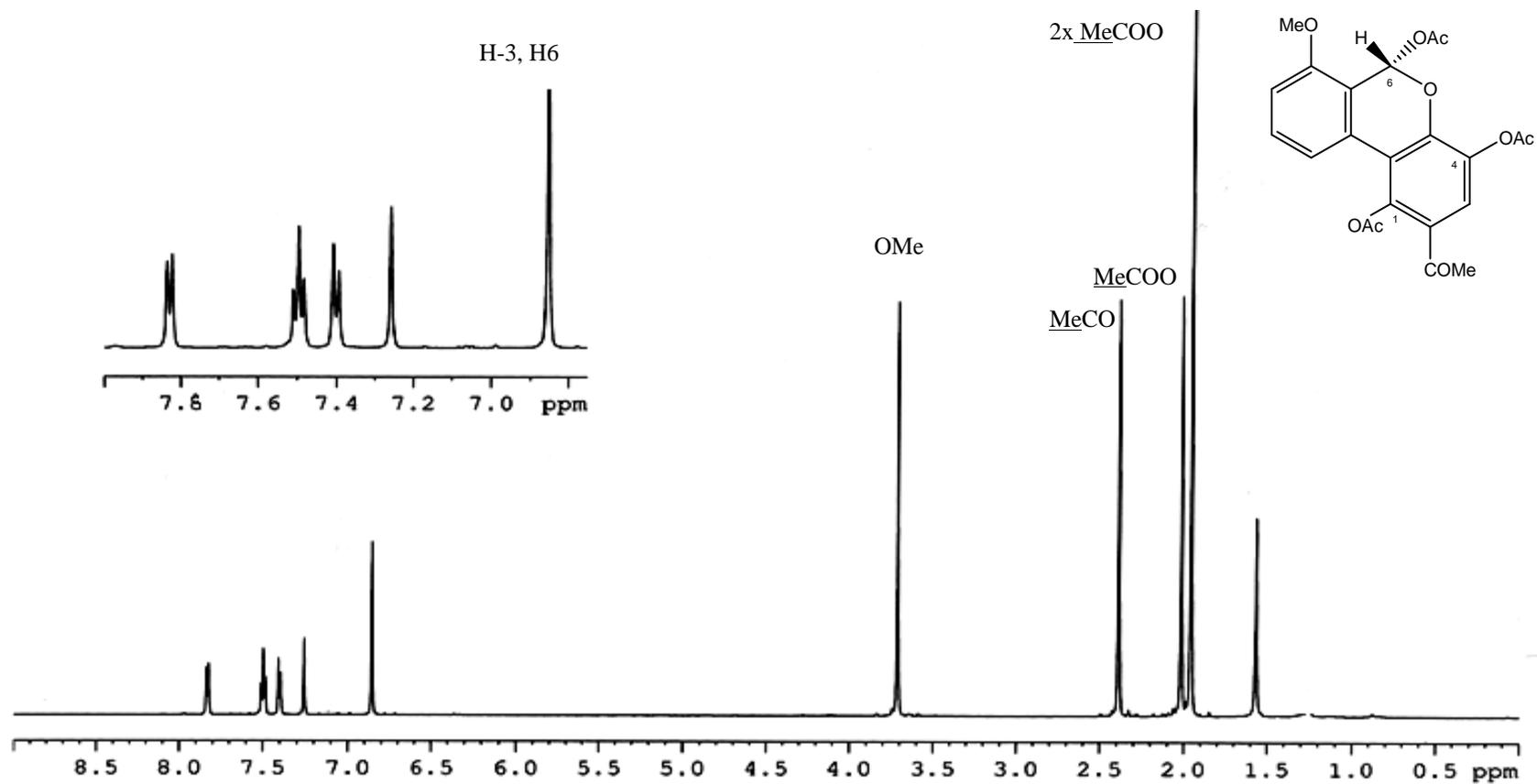


Fig. 23. ^1H NMR spectrum of triacetylalternethanoxin A recorded at 600 MHz.

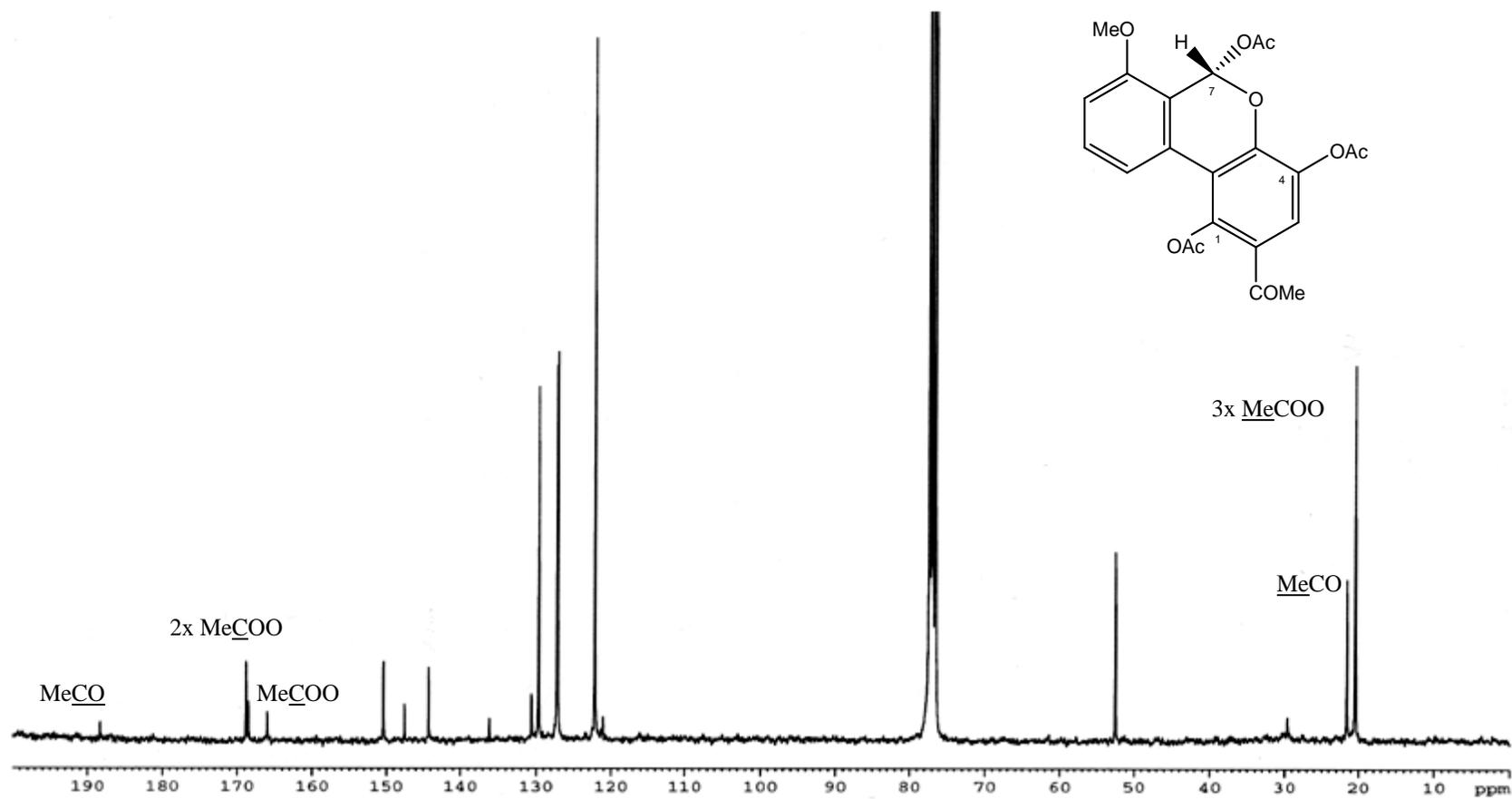


Fig. 24. ^{13}C NMR spectrum of triacetylalternethanoxin A recorded at 600 MHz.

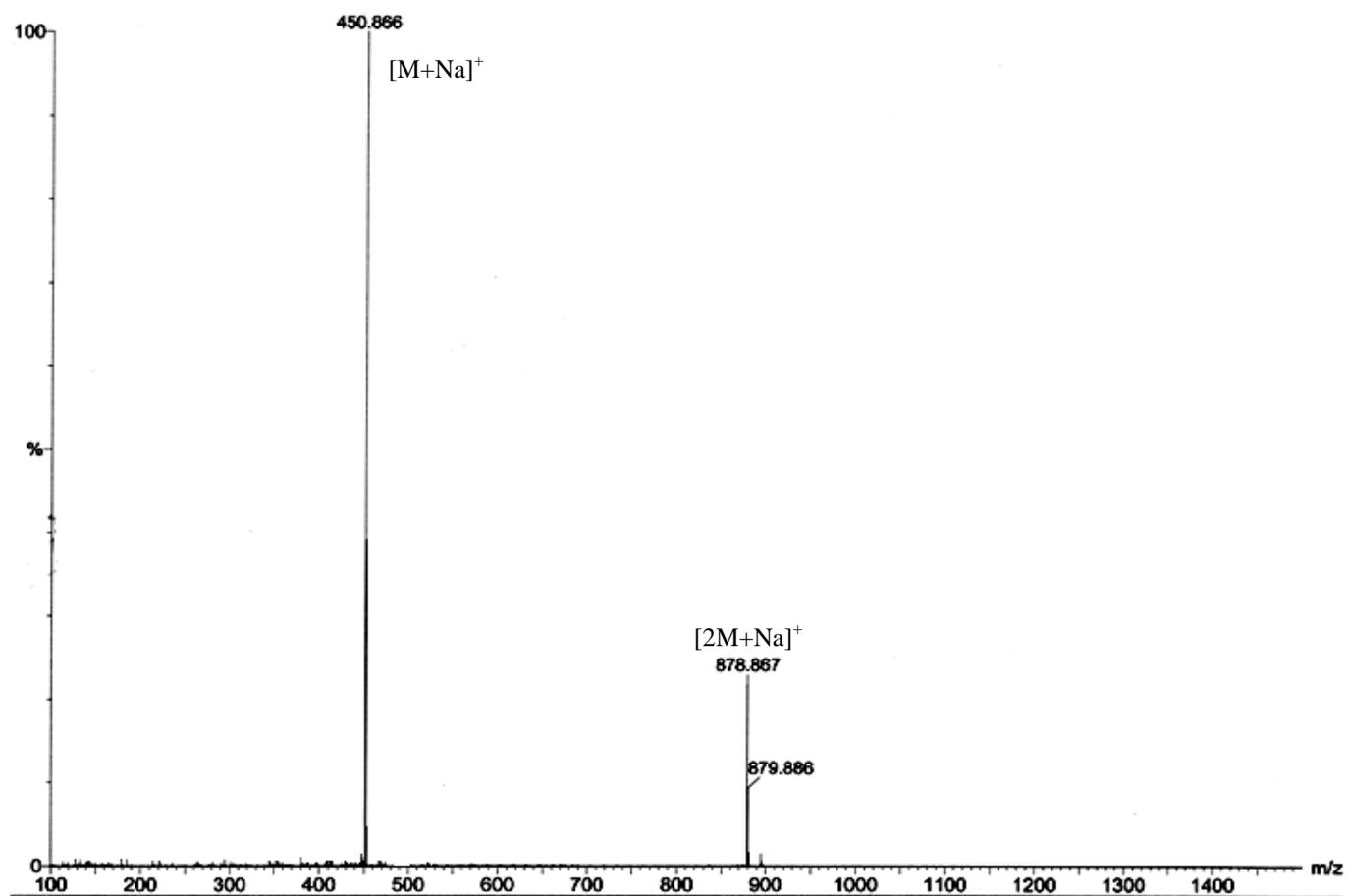


Fig. 25. ESI MS spectrum of triacetylalternethanoxin A recorded in positive modality.

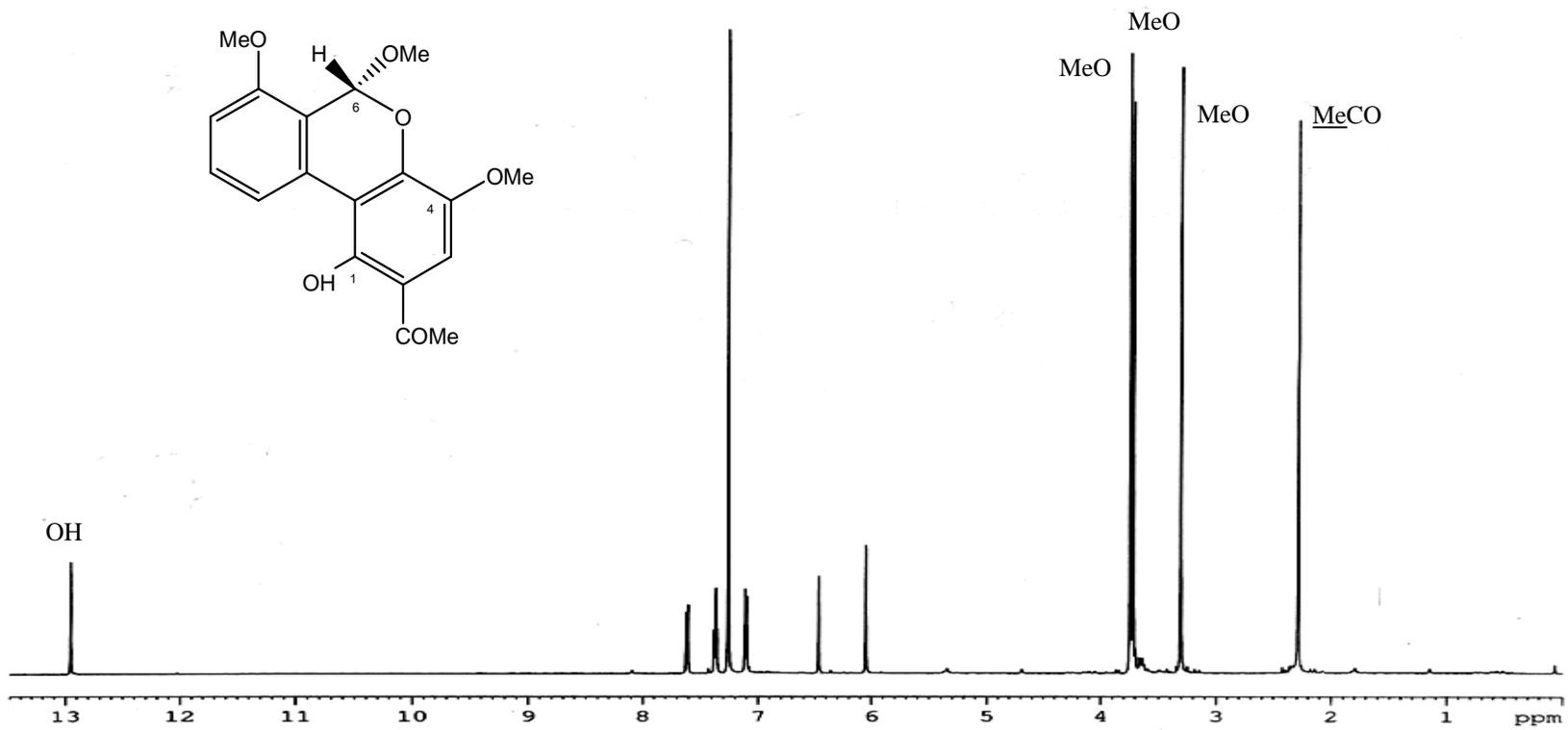


Fig. 26. ¹H NMR spectrum of alternethanoxin A dimethyl ether recorded at 600 MHz.

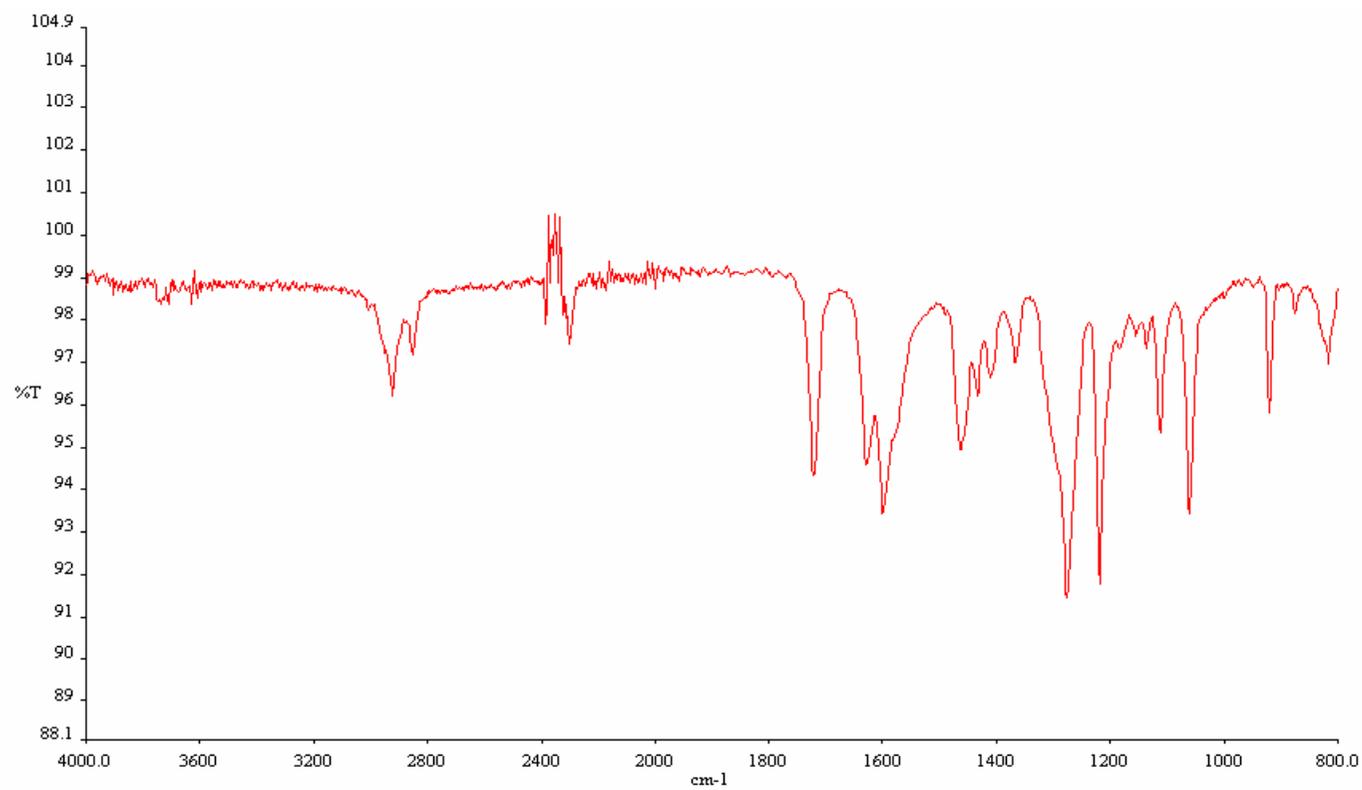


Fig. 27. IR spectrum of alternethanoxin A dimethyl ether deposited glassy film.

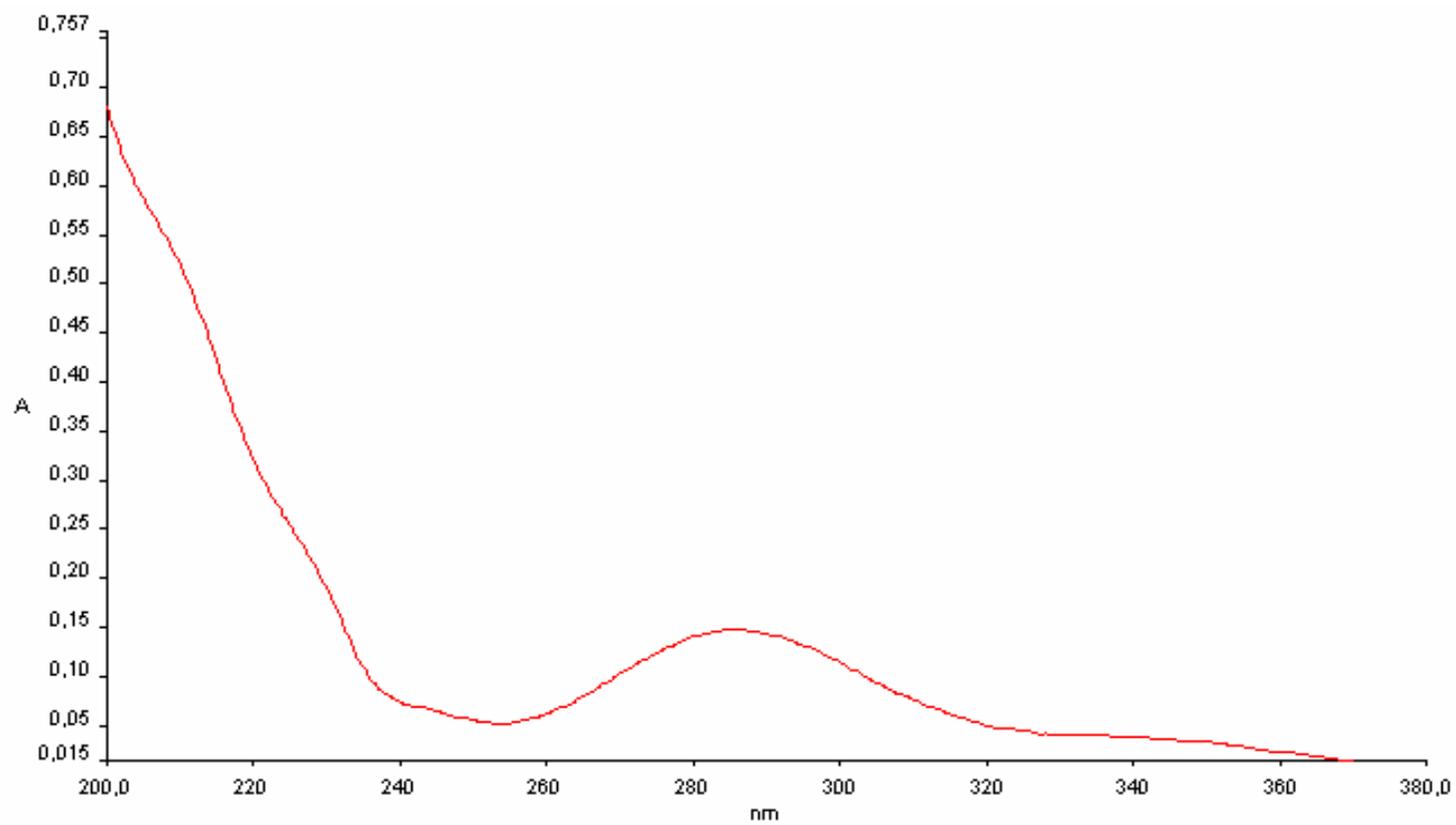


Fig. 28. UV spectrum of alternethanoxin A dimethyl ether recorded in MeCN solution.

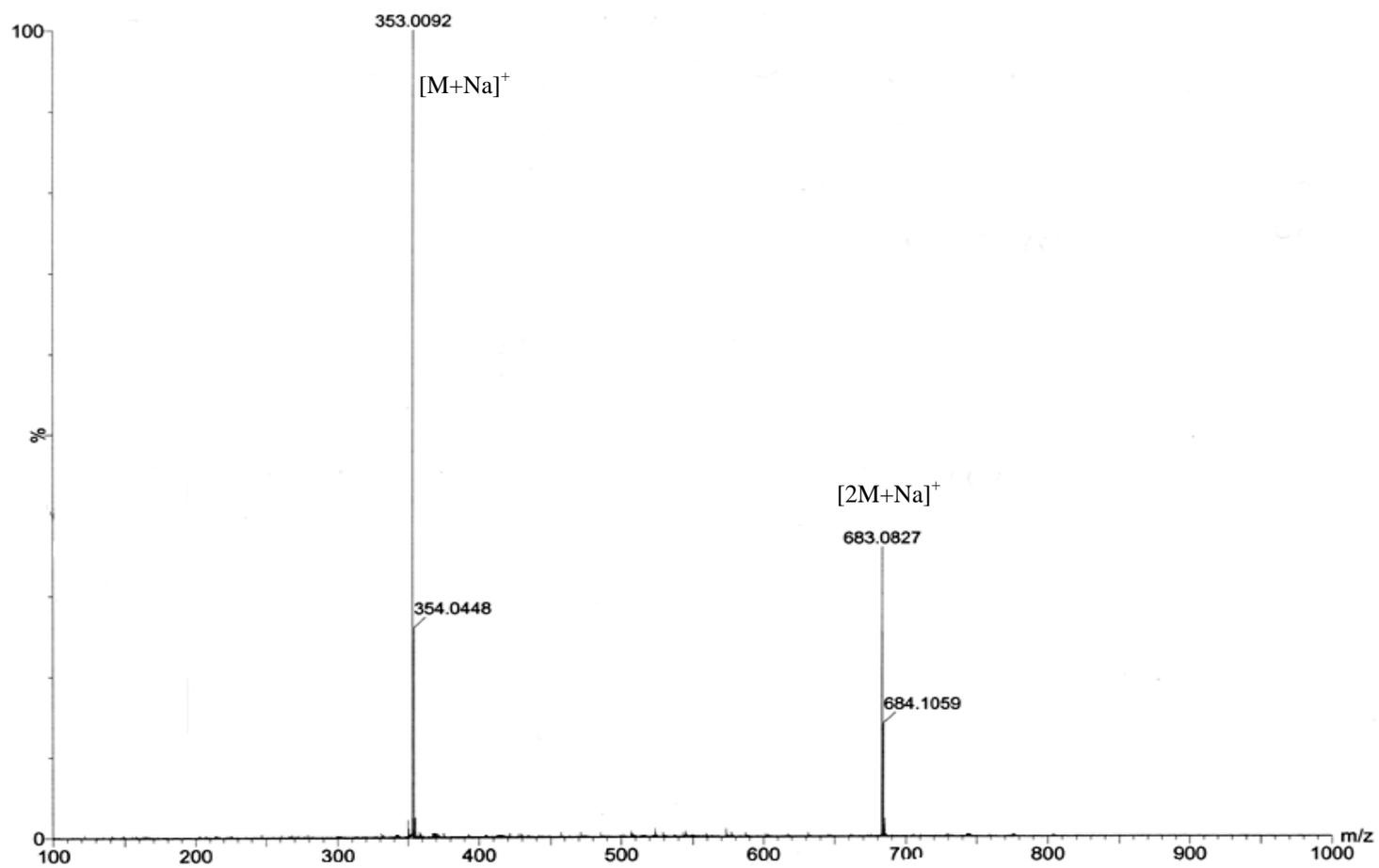


Fig. 29. ESI MS spectrum of alternethanoxin A dimethyl ether recorded in positive modality.

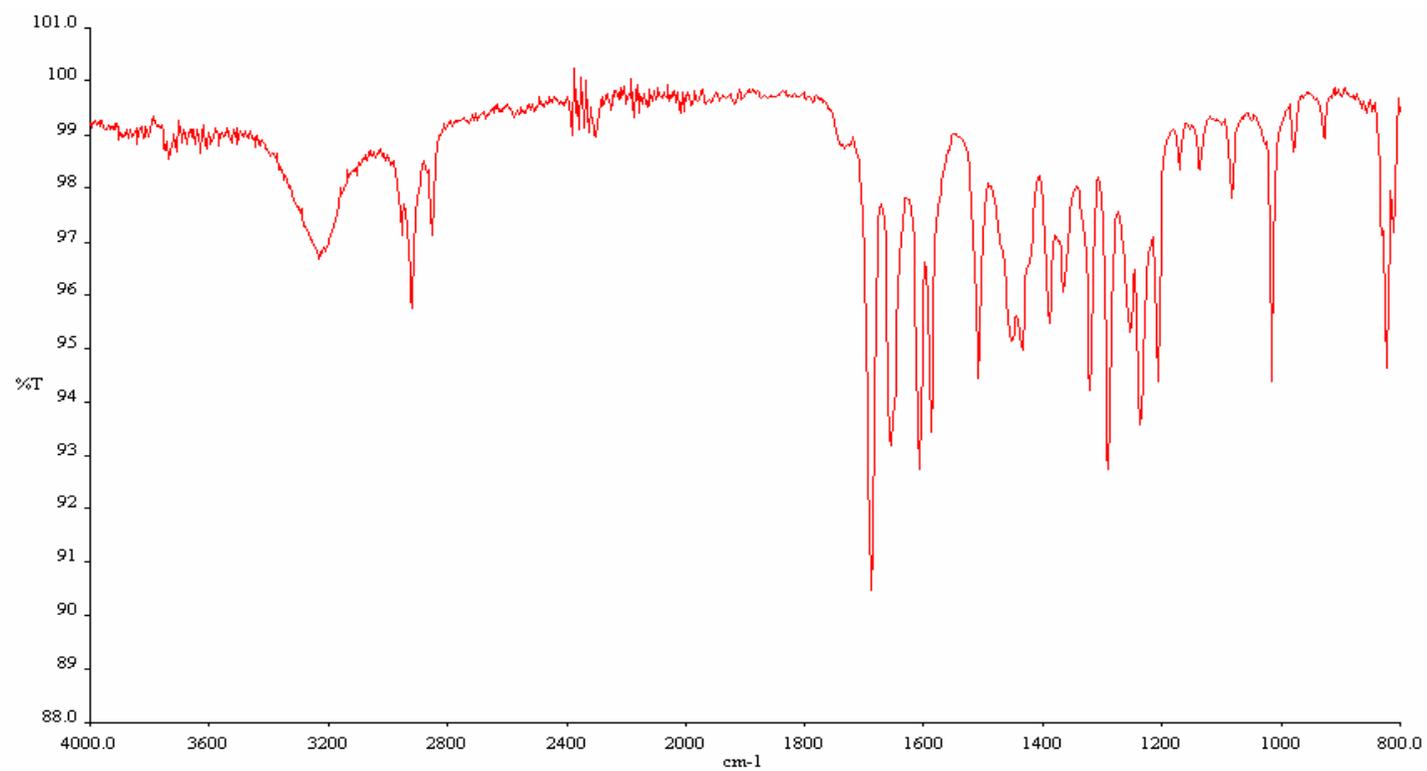


Fig. 30. IR spectrum of alternethanoxin B deposited glassy film.

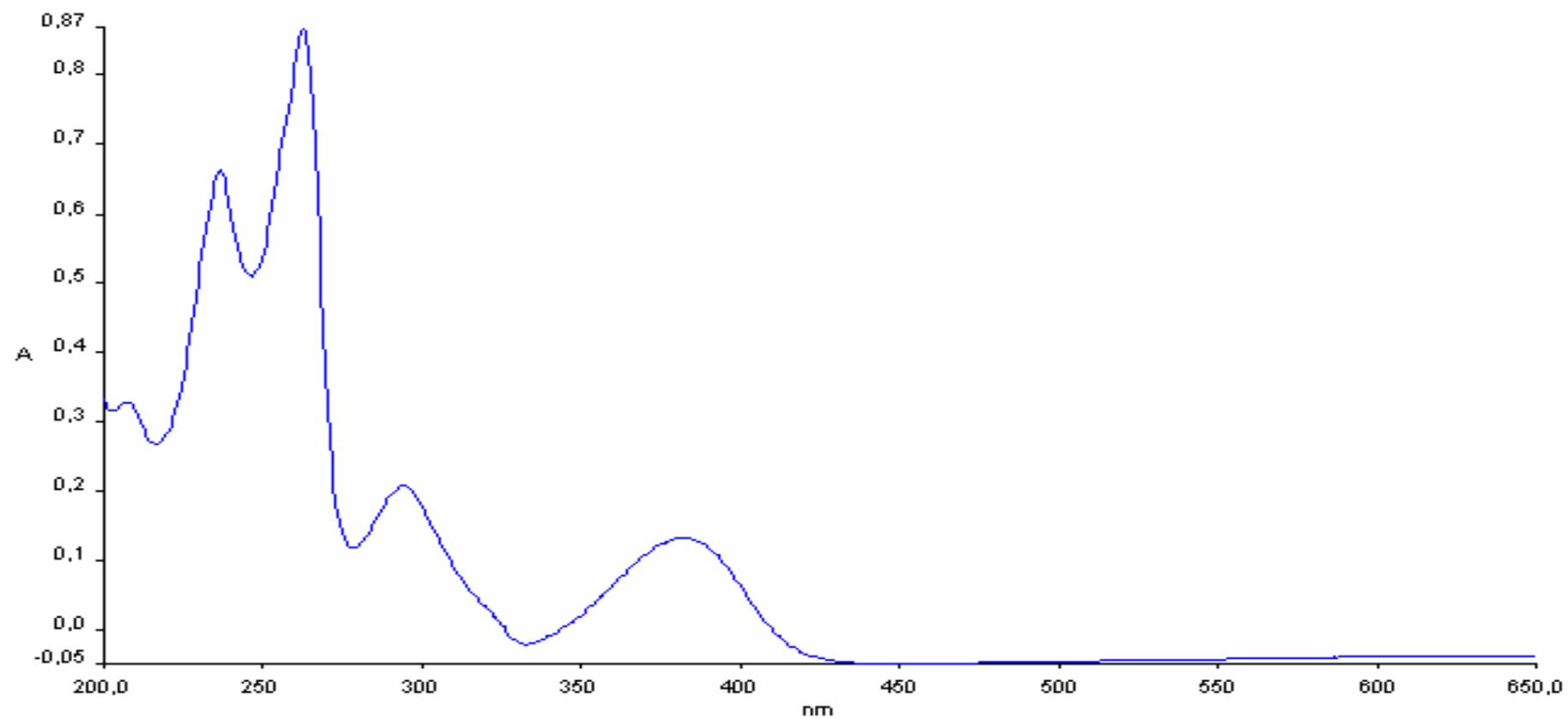


Fig. 31. UV spectrum of alternethanoxin B recorded in MeCN solution.

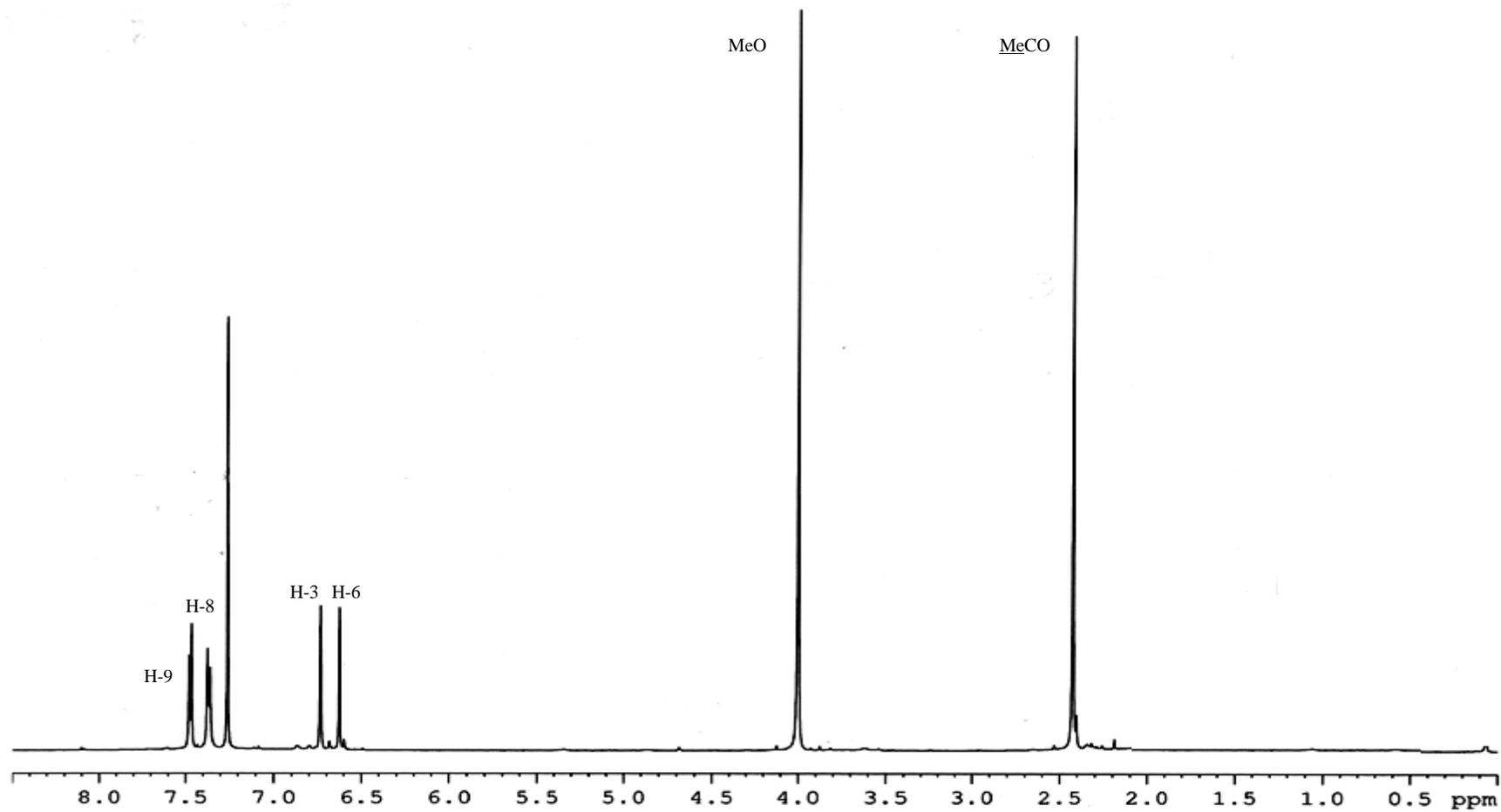


Fig. 32. ^1H NMR spectrum of alternethanoxin B recorded at 600 MHz.

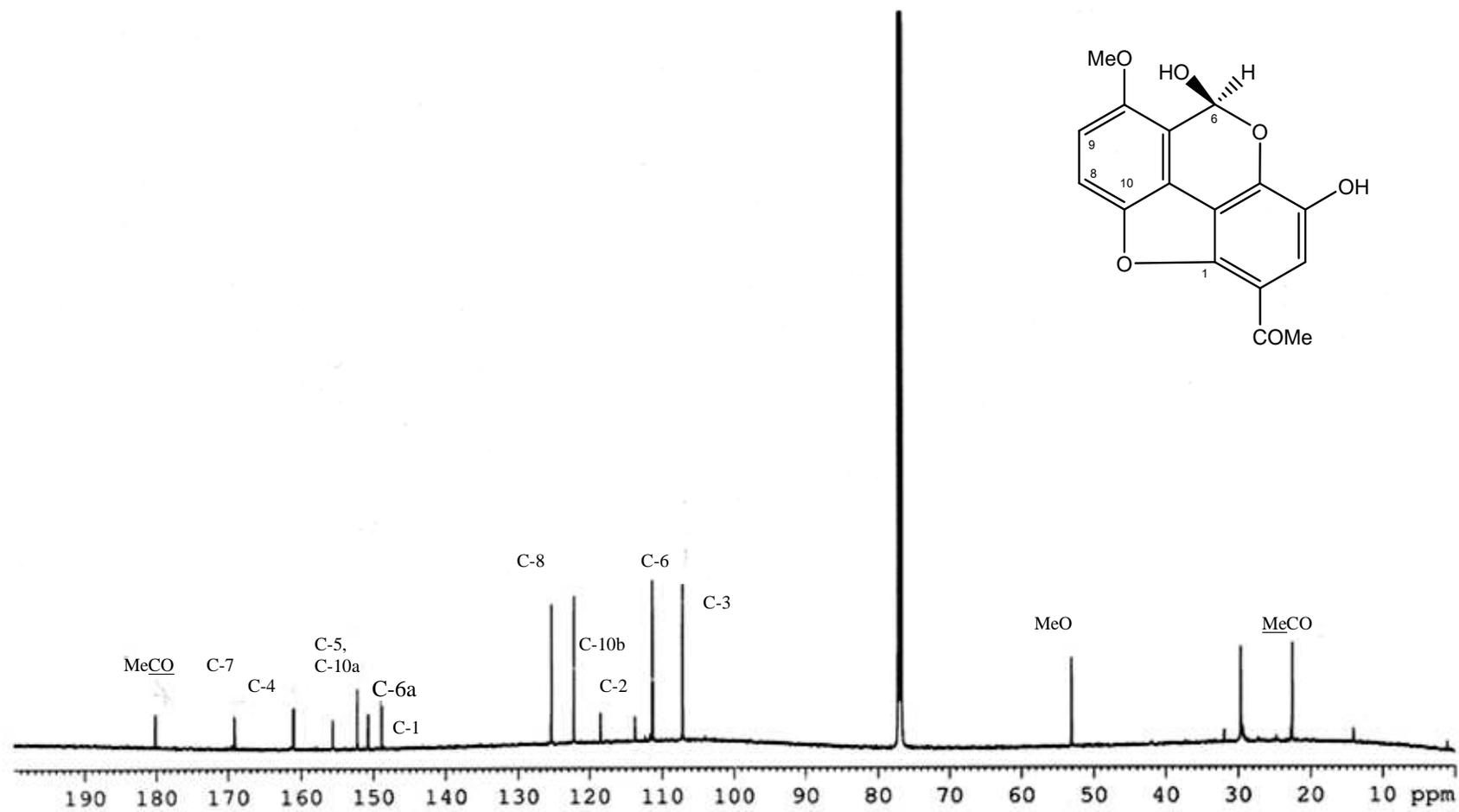


Fig. 33. ^{13}C NMR spectrum of alternethanoxin B recorded at 600 MHz.

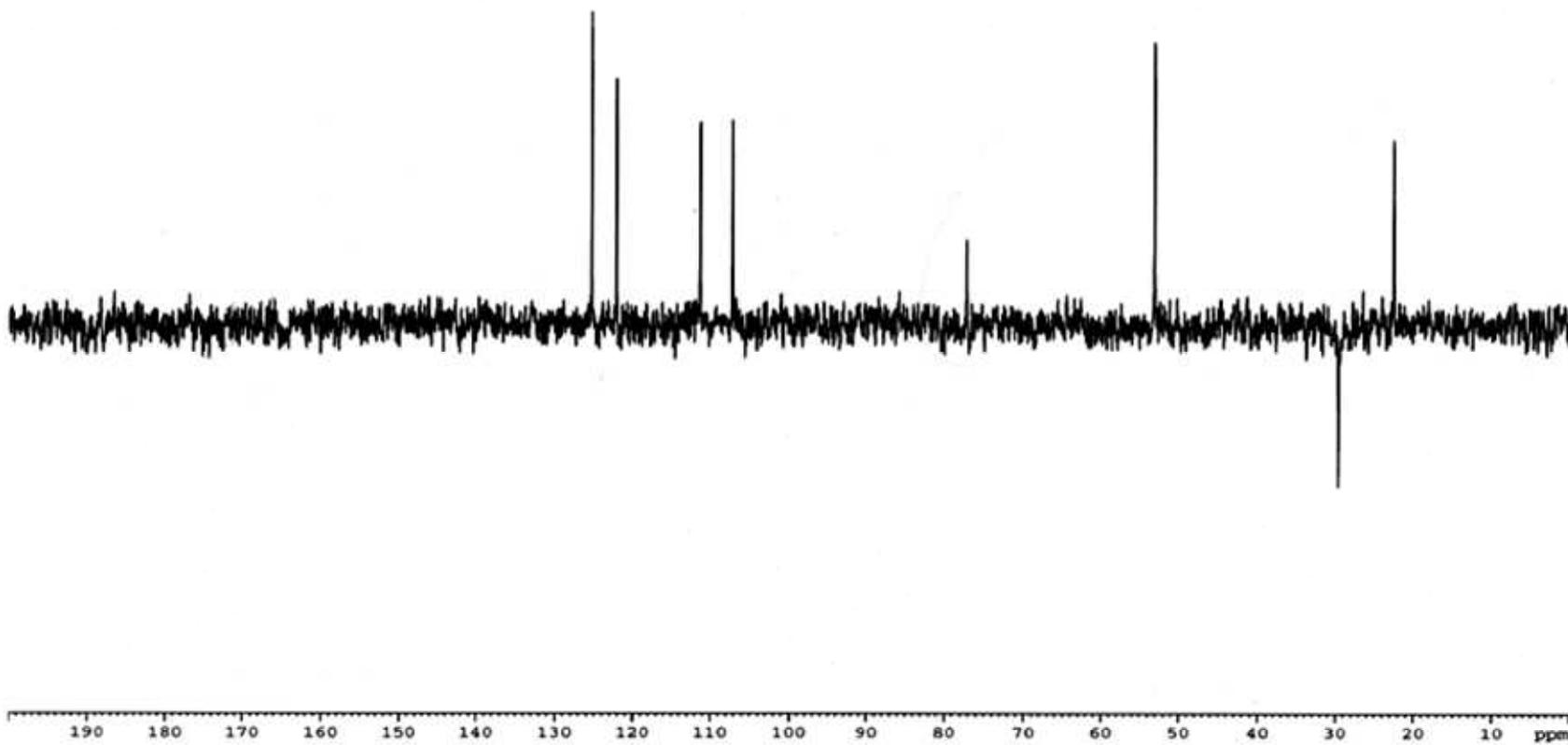


Fig. 34. DEPT spectrum of alternethanoxin B recorded at 600 MHz.

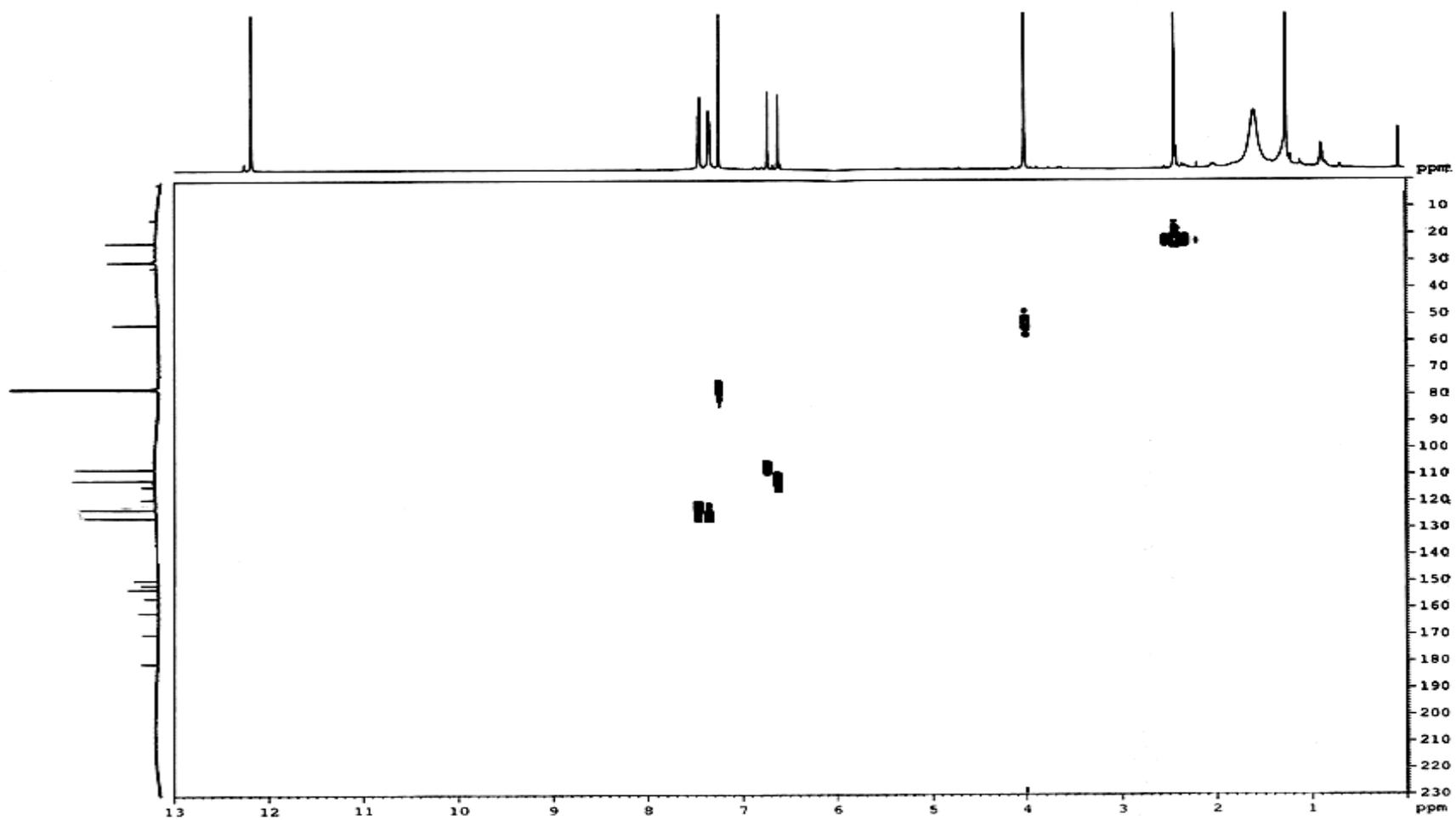


Fig. 35. HSQC spectrum of alternethanoxin B recorded at 600 MHz.

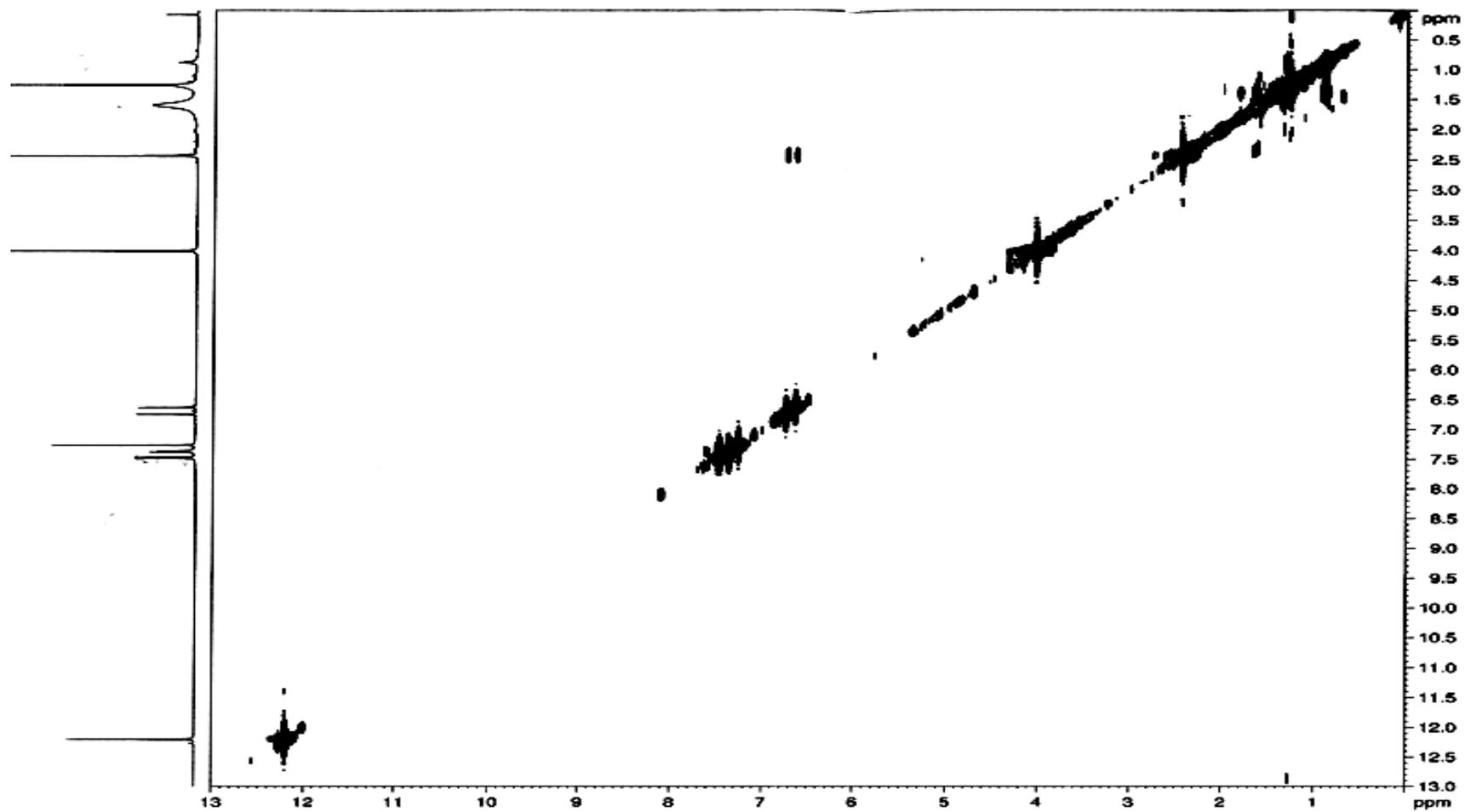


Fig. 36. COSY spectrum of alternethanoxin B recorded at 600 MHz.

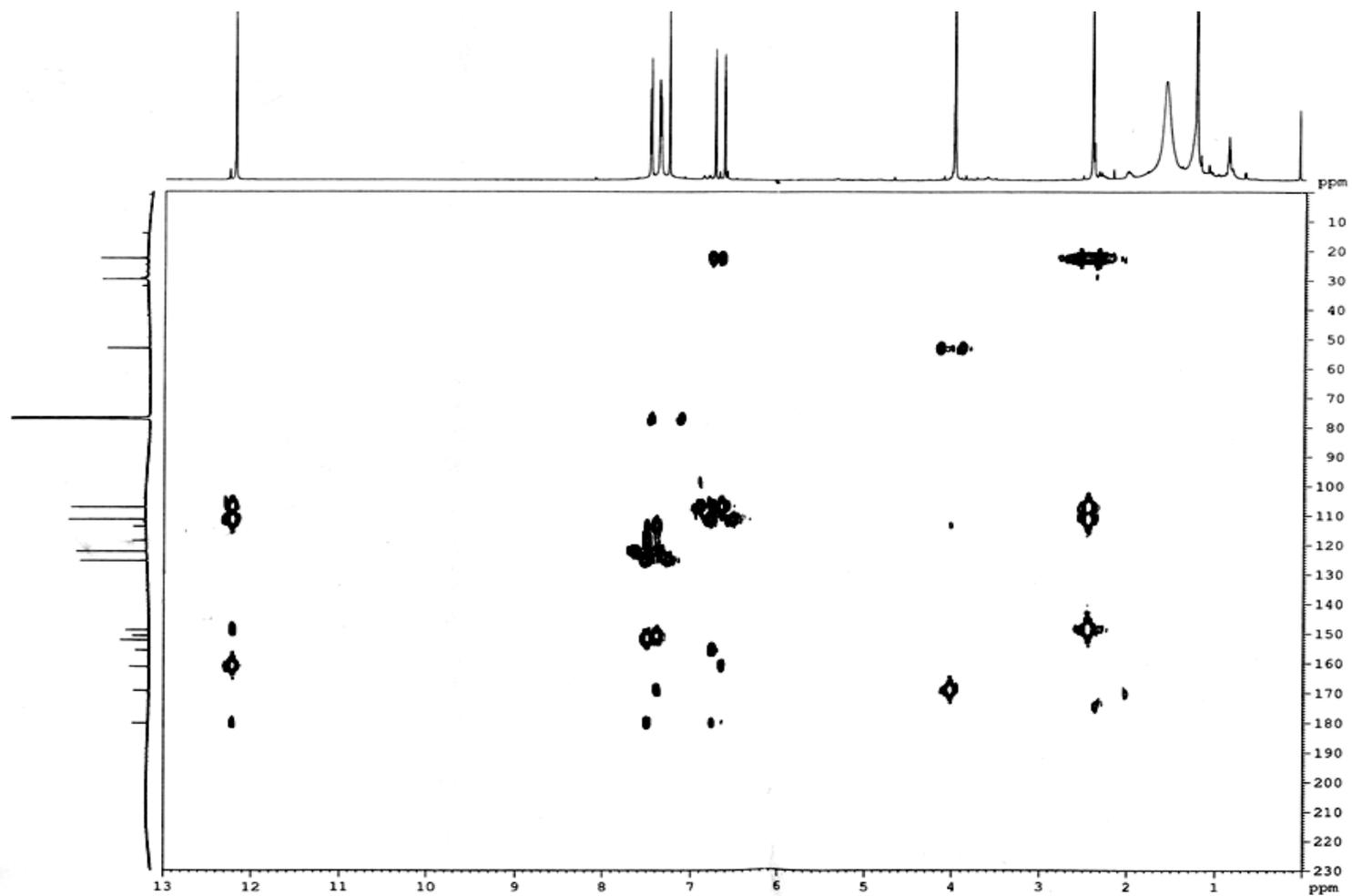


Fig. 37. HMBC spectrum of alternethanoxin B recorded at 600 MHz.

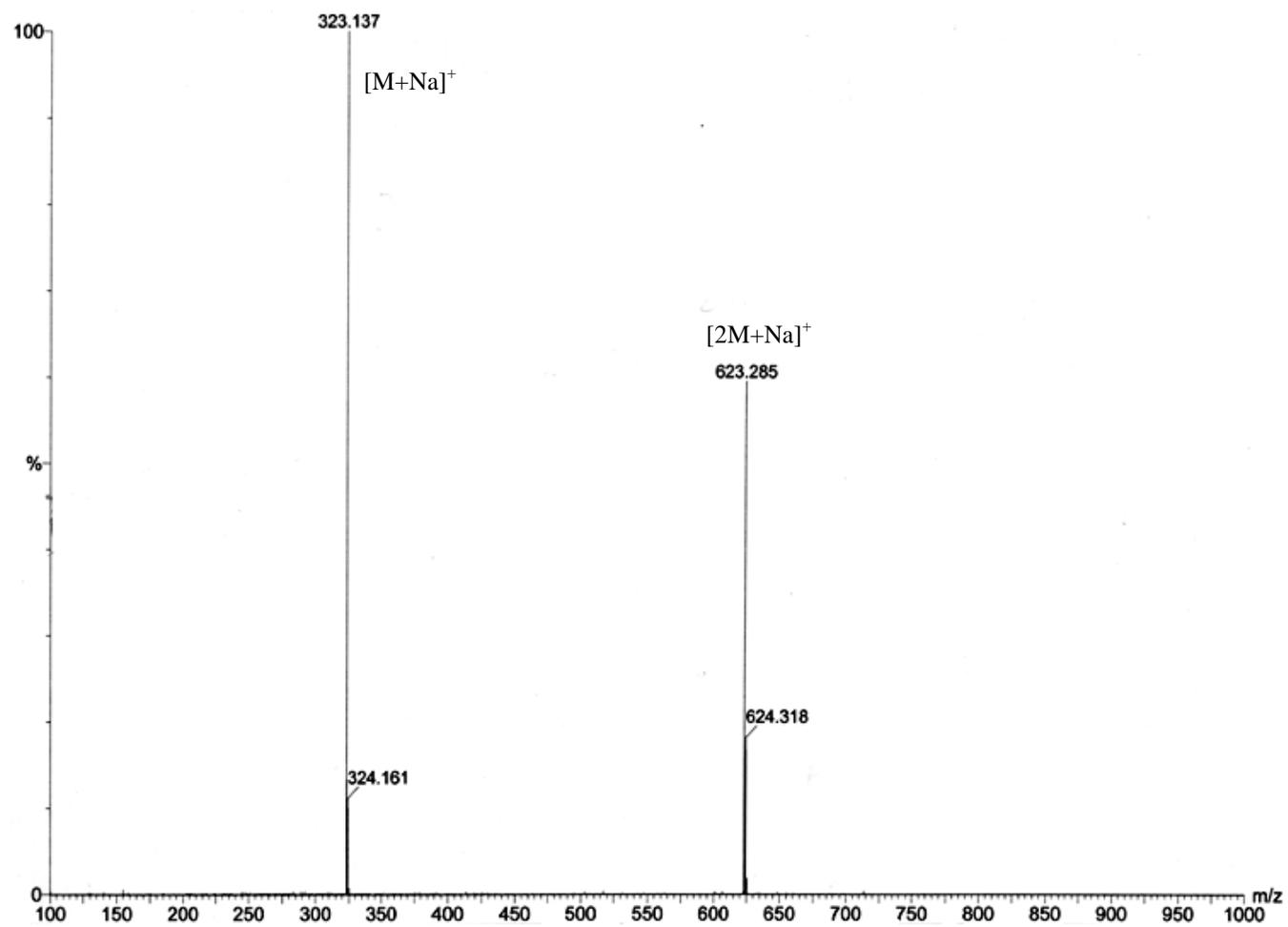


Fig. 38. ESI MS spectrum of alternethanoxin B recorded in positive modality.

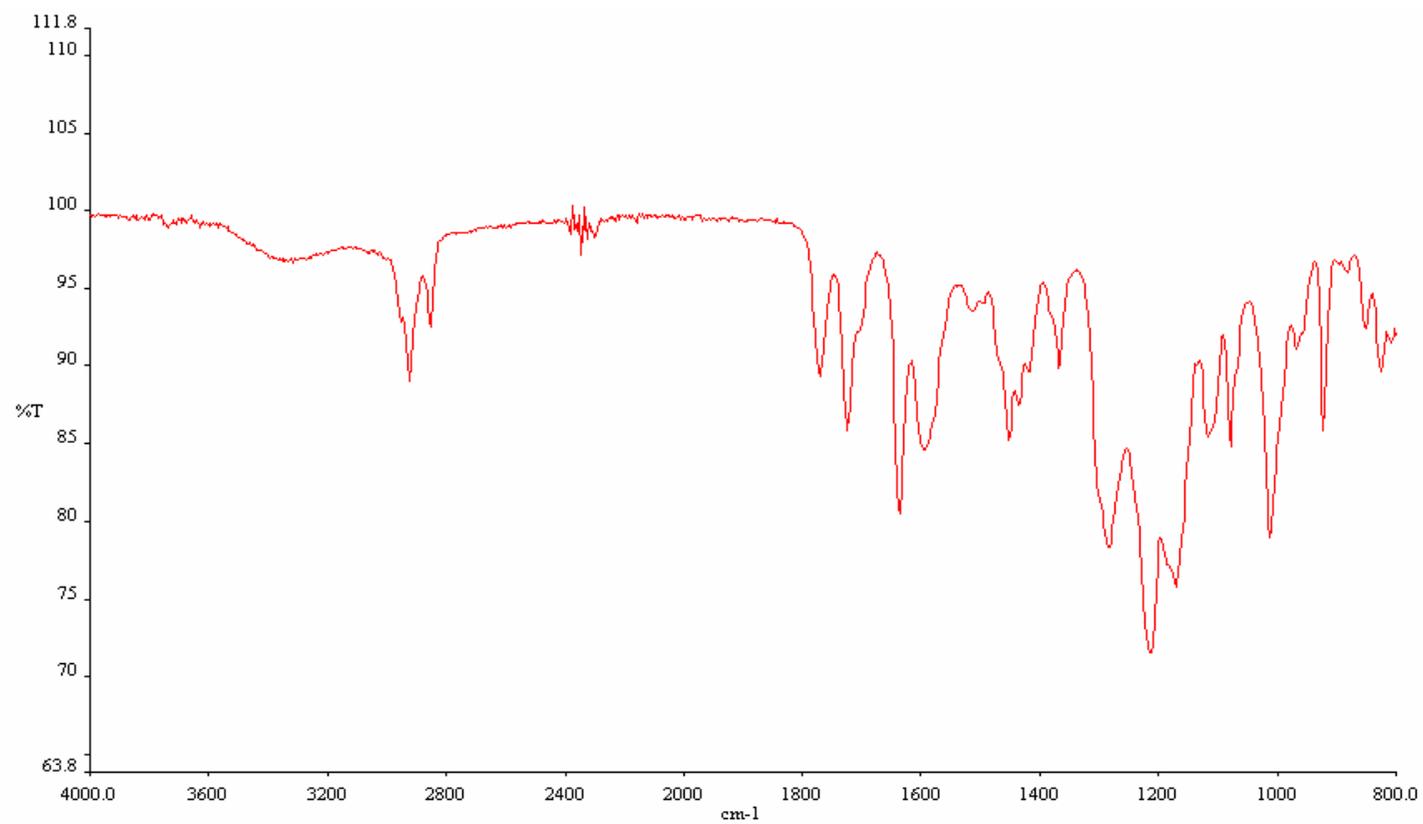


Fig. 39. IR spectrum of *S*-MTPA ester of alternethanoxin A deposited glassy film.

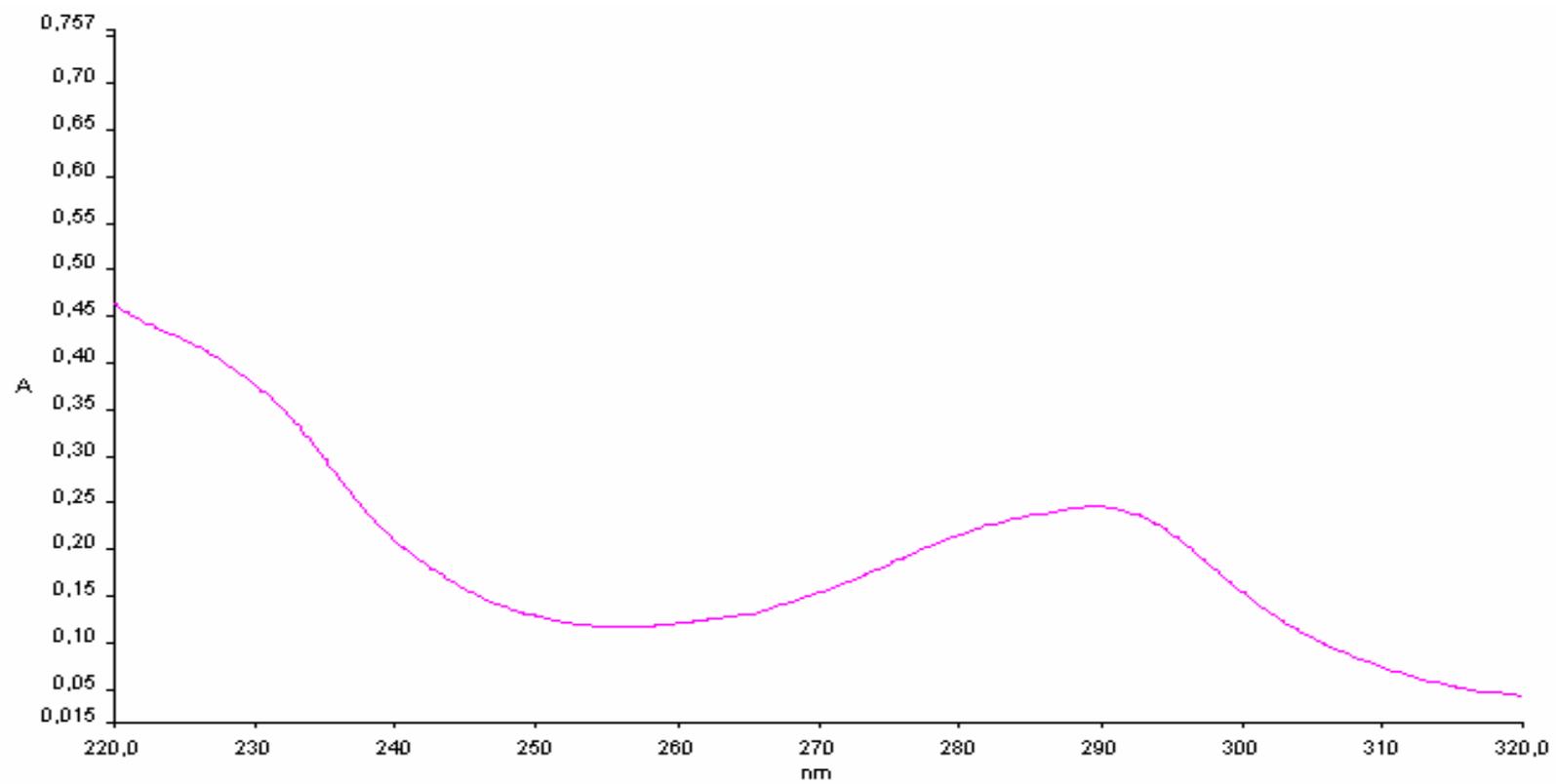


Fig. 40. UV spectrum of *R*-MTPA triester of alternethanoxin A recorded in MeCN solution.

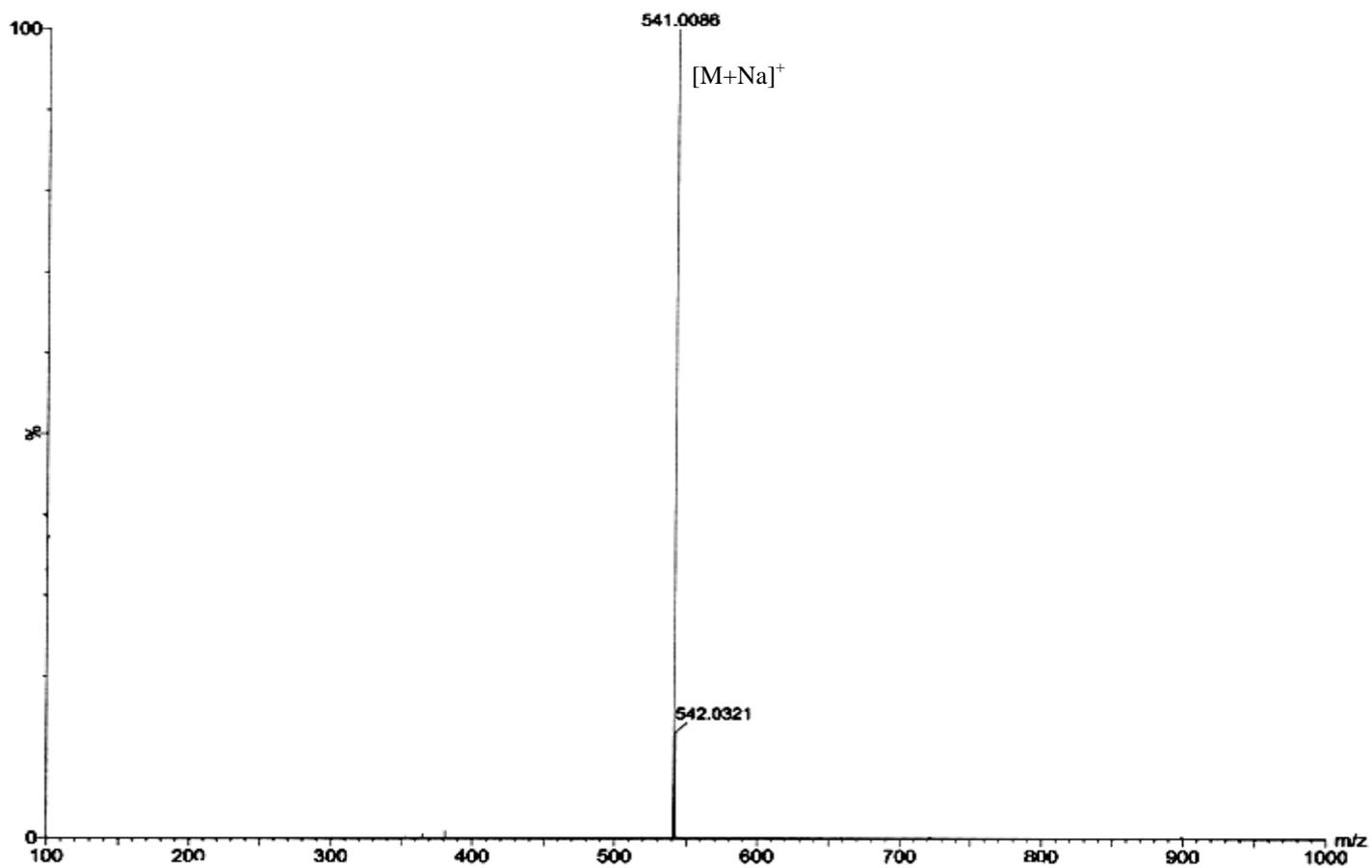


Fig. 41. ESI MS spectrum of *S*-MTPA ester of alternethanoxin A recorded in positive modality.

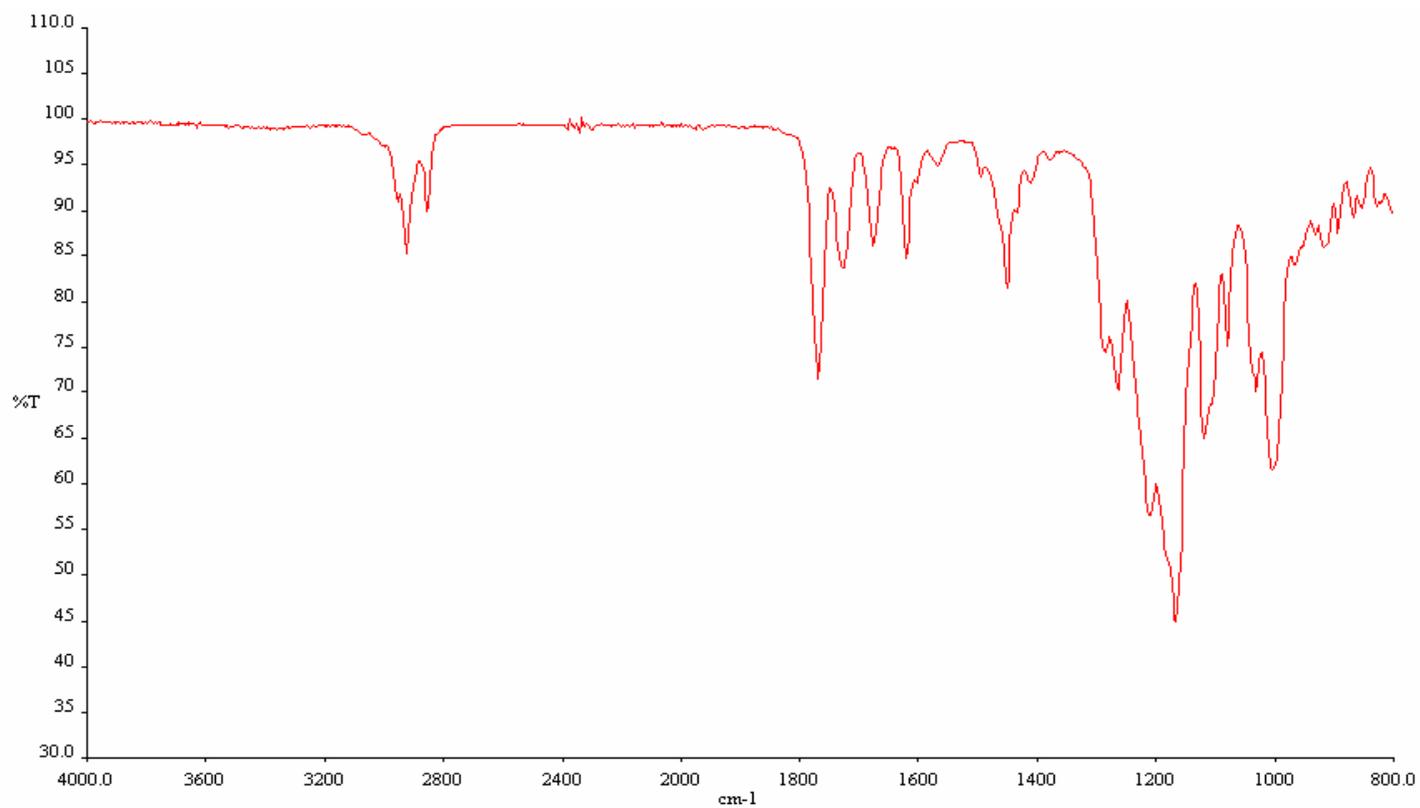


Fig. 42. IR spectrum of *R*-MTPA triester of alternethanoxin A deposited glassy film.

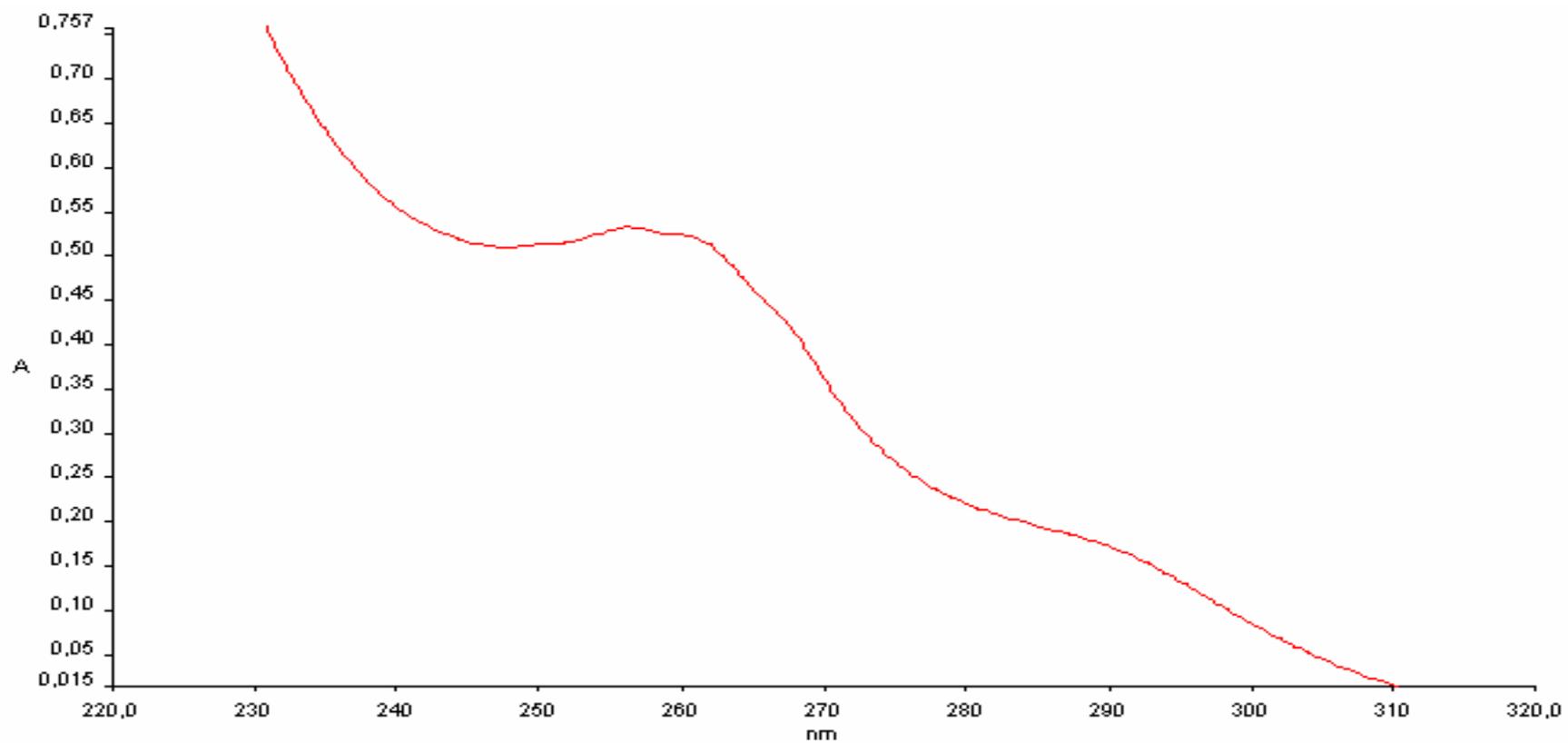


Fig. 43. UV spectrum of *S*-MTPA ester of alternethanoxin A recorded in MeCN solution.

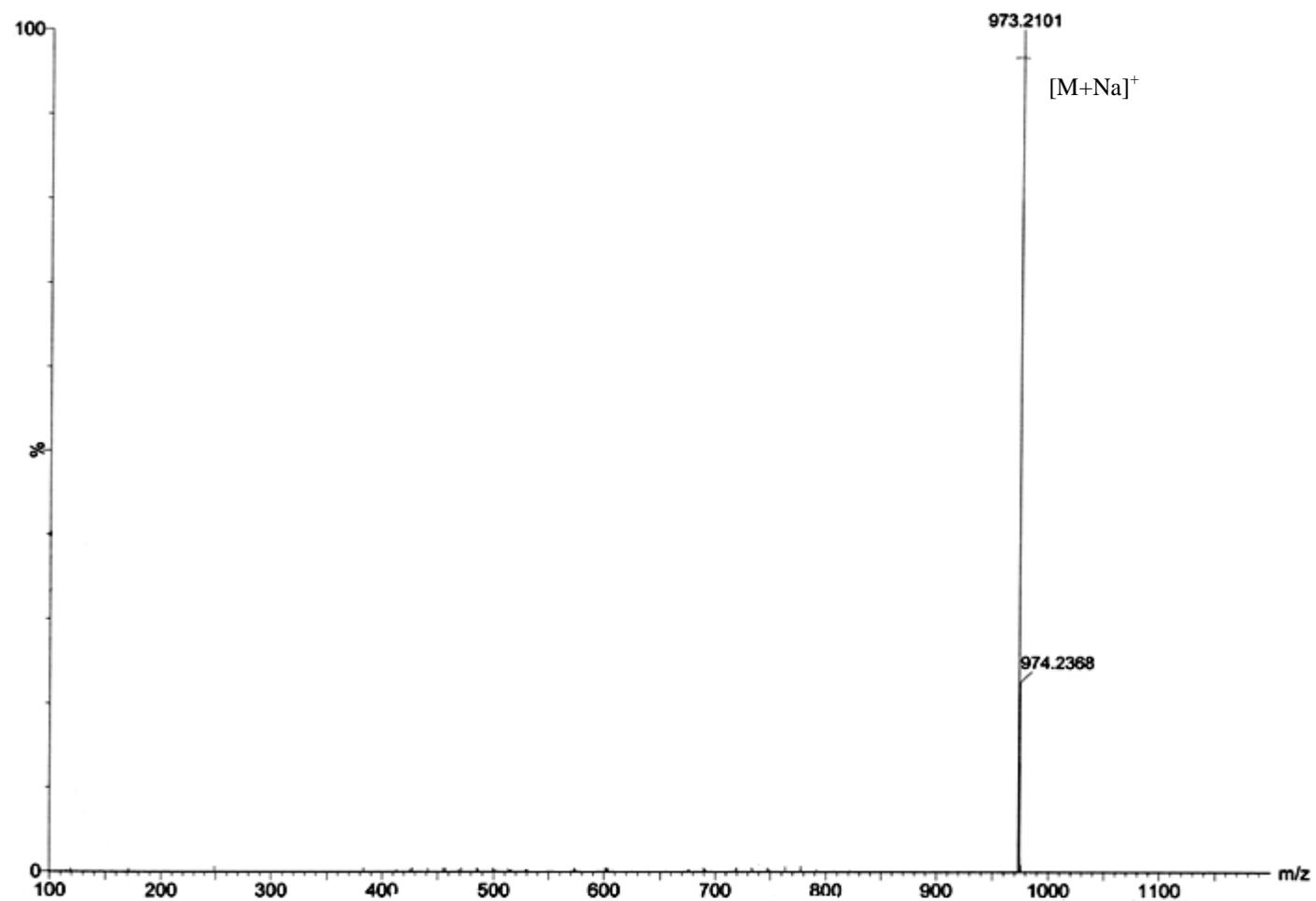


Fig. 44. ESI MS spectrum of *R*-MTPA triester of alternethanoxin A recorded in positive modality.

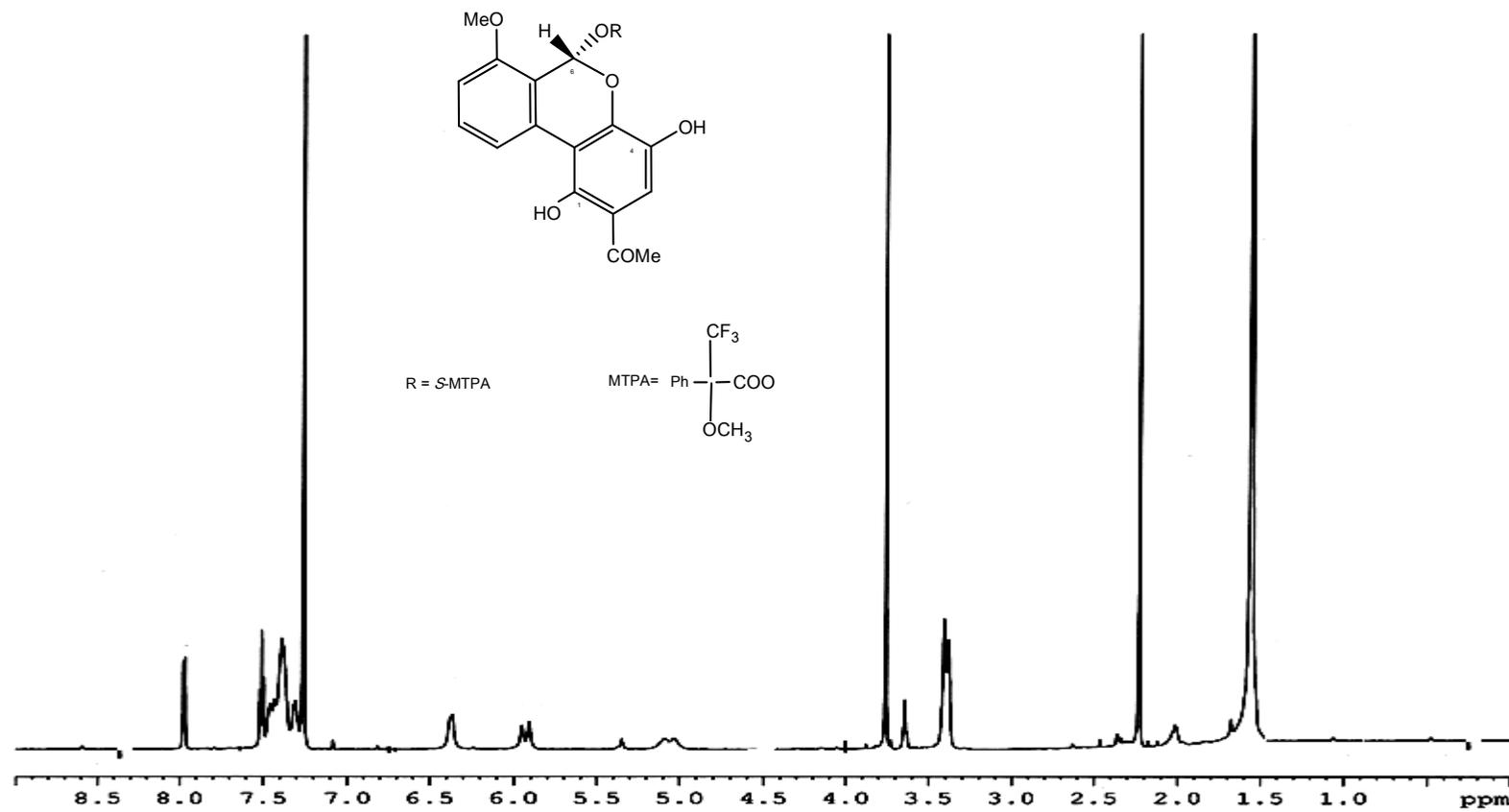


Fig. 45. ^1H NMR spectrum of *S*-MTPA ester of alternethanoxin A recorded at 600 MHz.

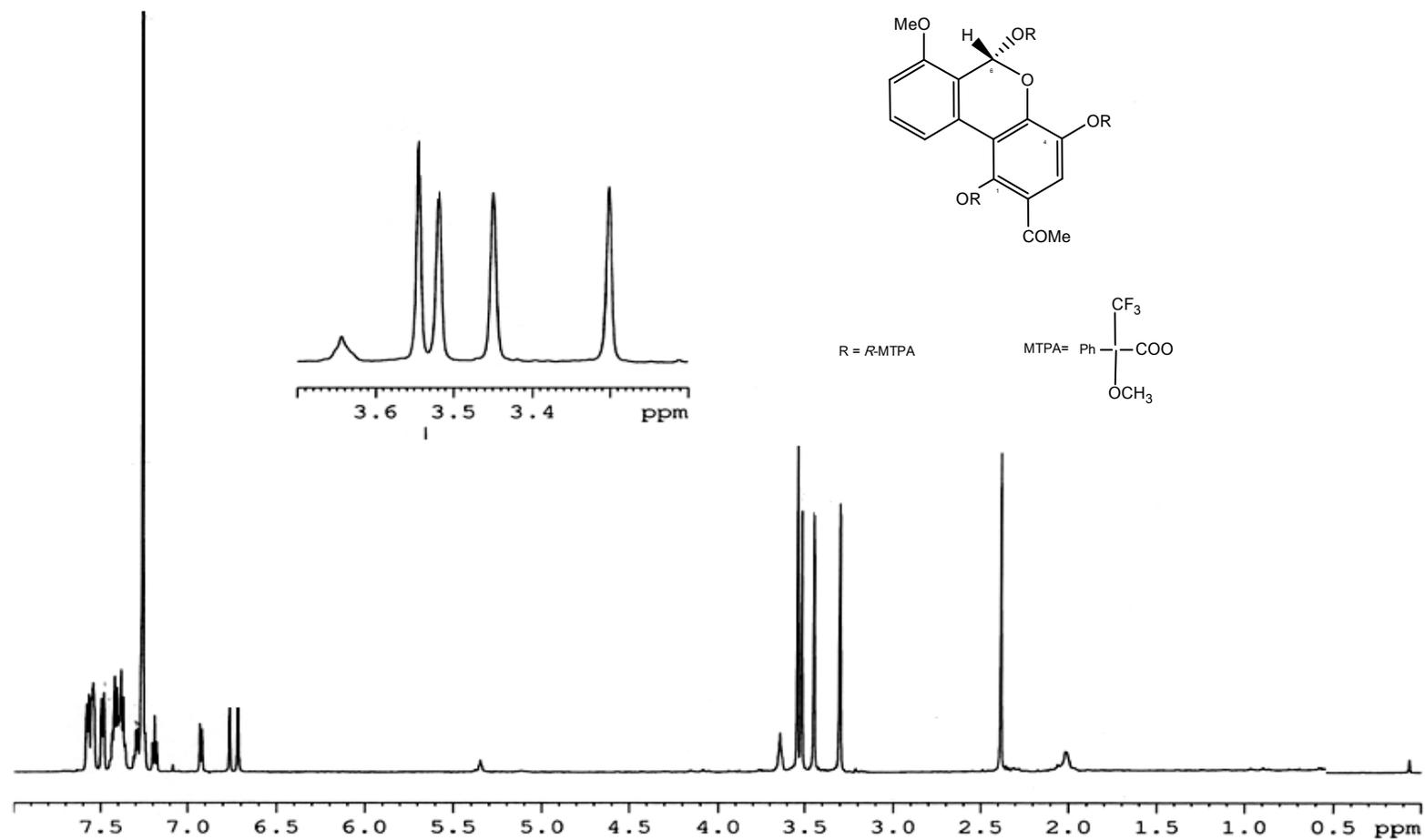
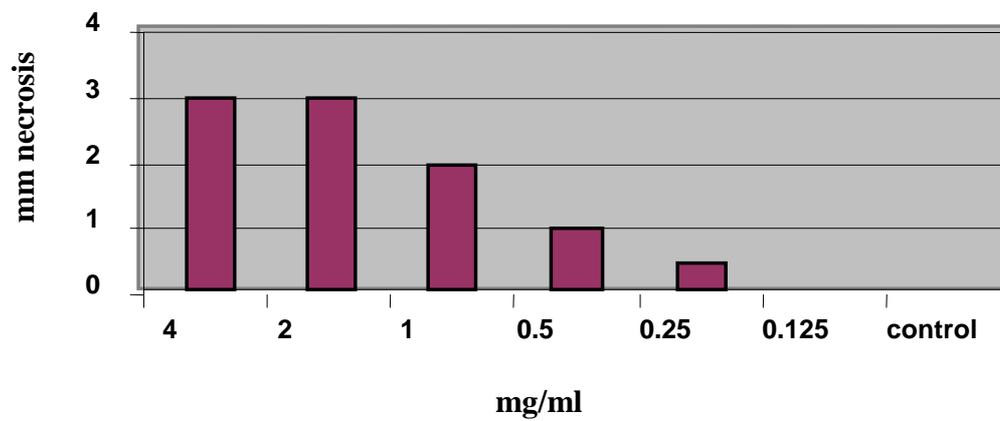


Fig. 46. ^1H NMR spectrum of *R*-MTPA triester of alternethanoxin A recorded at 600 MHz.

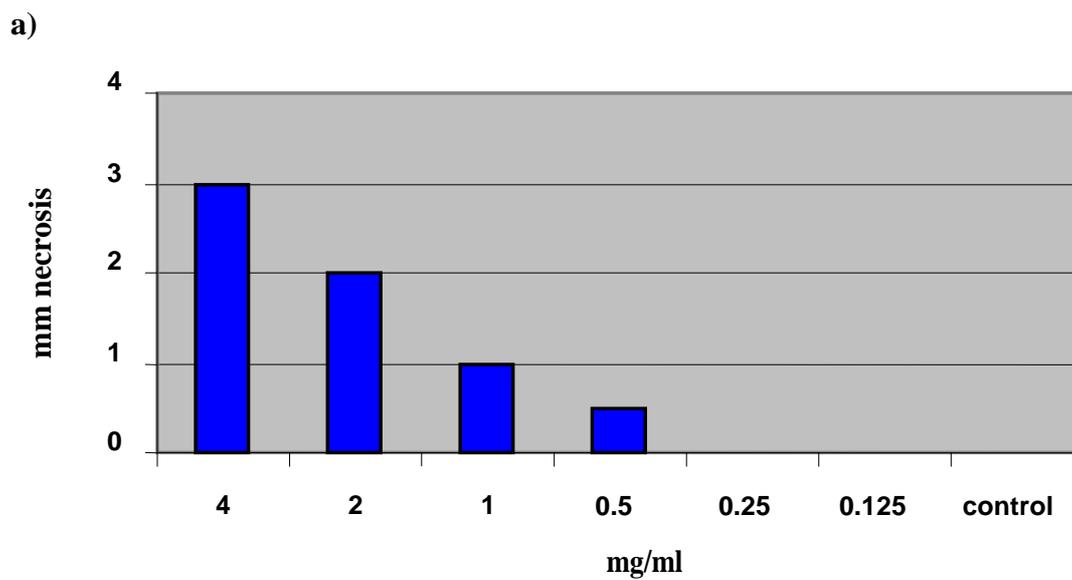
a)



b)



Fig. 47a and 48b. Effect of concentration of alternethanoxin A on size of necrotic lesion on *S. arvensis* leaf discs.



b)

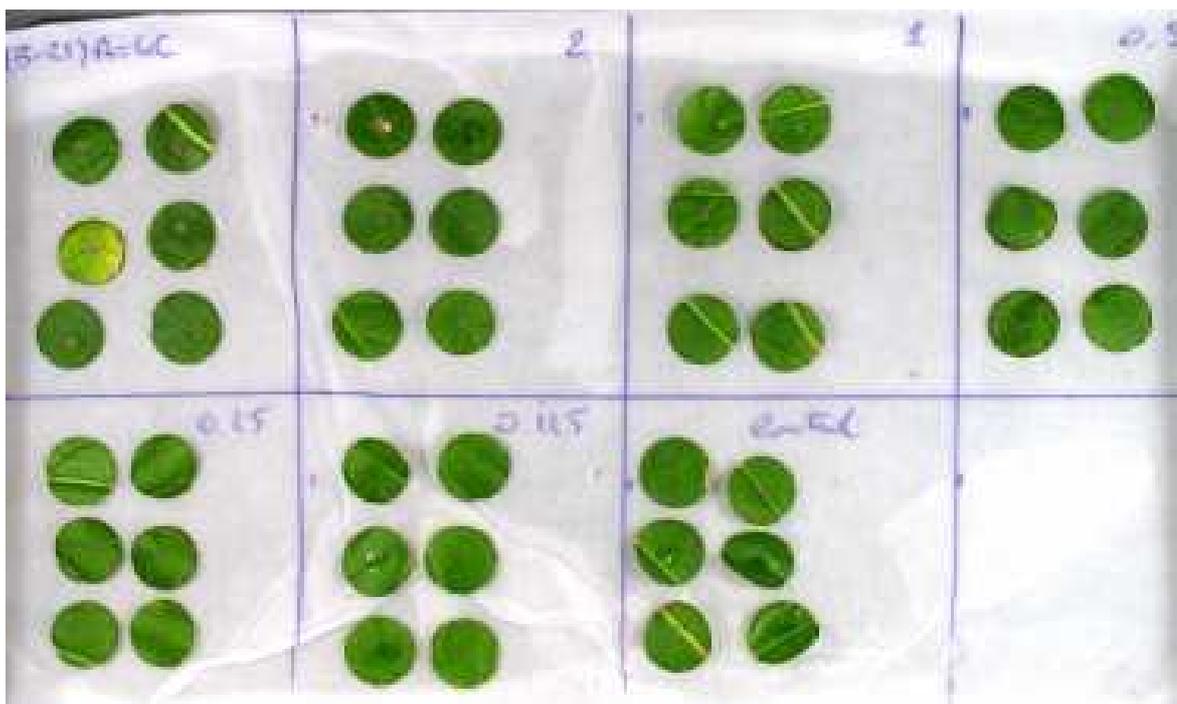
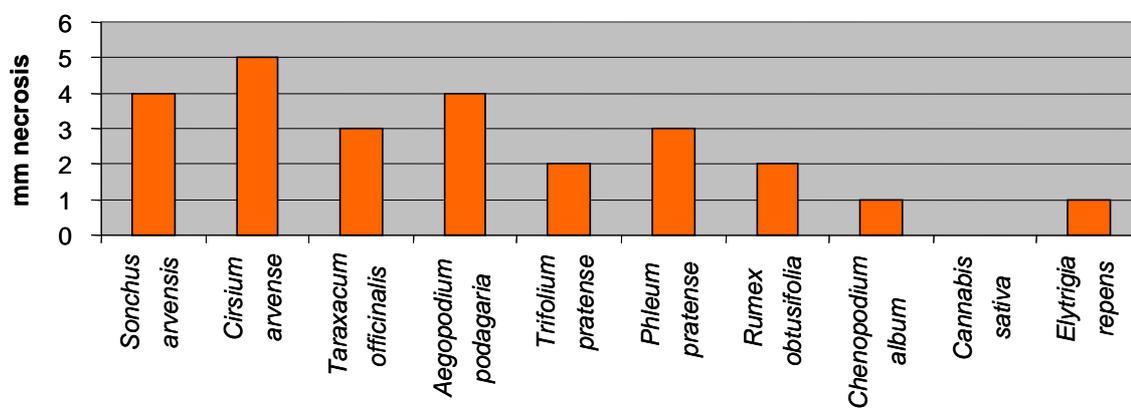


Fig. 48a and 48b. Effect of concentration of alternethanoxin B on size of necrotic lesion on *S. arvensis* leaf discs.

a)



b)

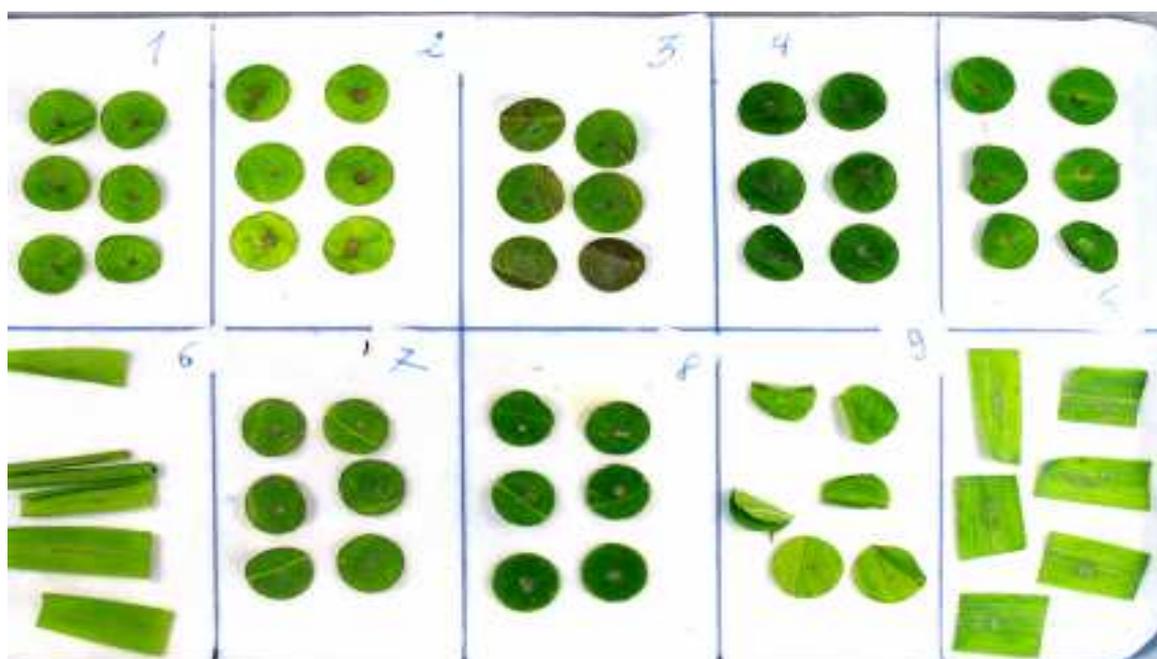


Fig. 49a and 49b. Host range assay of alternethanoxins A (1= *Sonchus arvensis*, 2= *Cirsium arvense*, 3= *Taraxacum officinalis*, 4= *Aegopodium podagaria*, 5= *Trifolium pratense*, 6 =*Phleum pratense*, 7= *Rumex obtusifolia*, 8 =*Chenopodium album*, 9= *Cannabis sativa*, 10=*Elytrigia repens*).

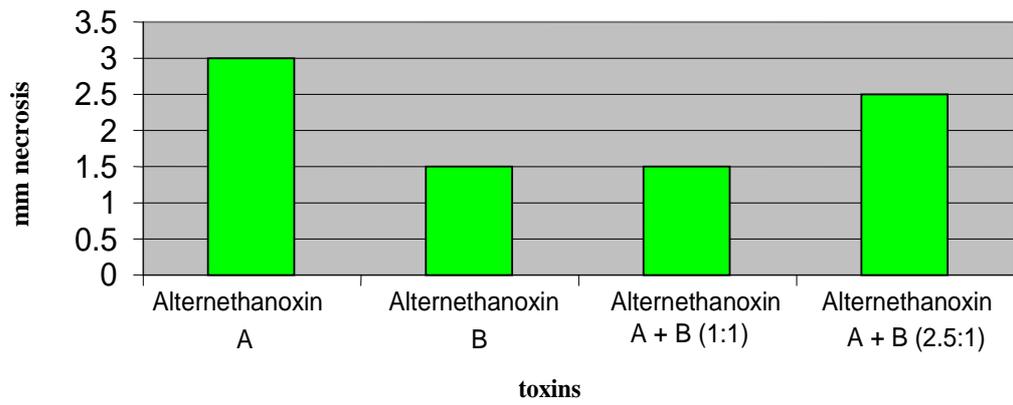


Fig. 50. Effect of combined application of alternethanoxins A and B

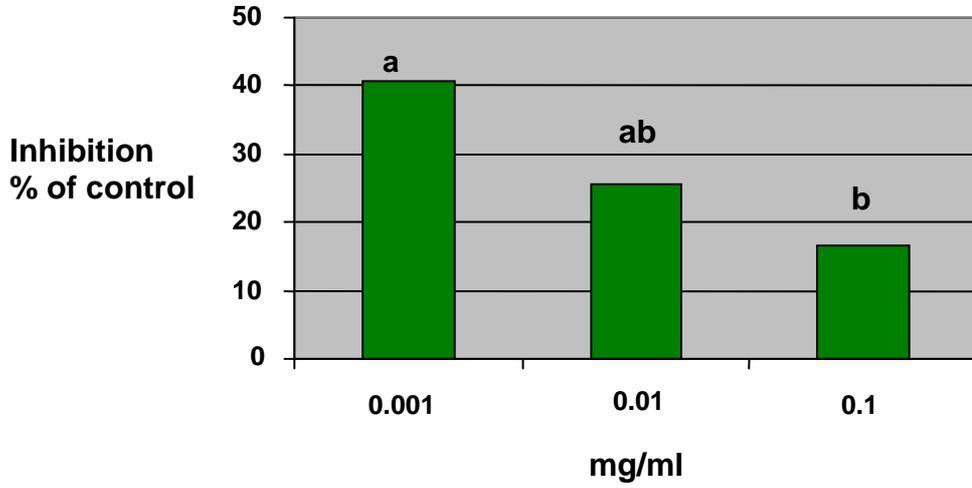


Fig. 51. Effect of alternethanoxin A tested at different concentration on root growth in seedlings of lettuce (24 h post application). Means marked with same letter are not differed significantly at $p=0.05$ by Fischer's LSD test

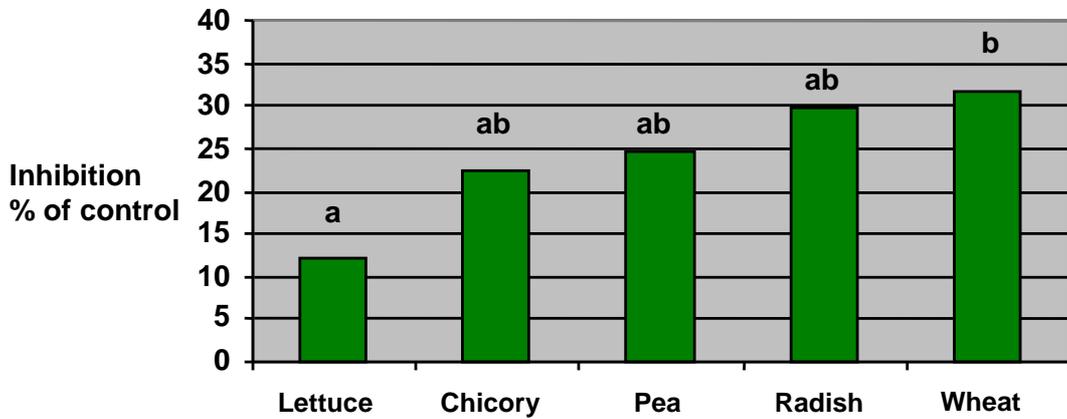


Fig. 52. Effect of alternethanoxin A on root growth in seedlings of different plants at the concentration 0.05 mg/ml

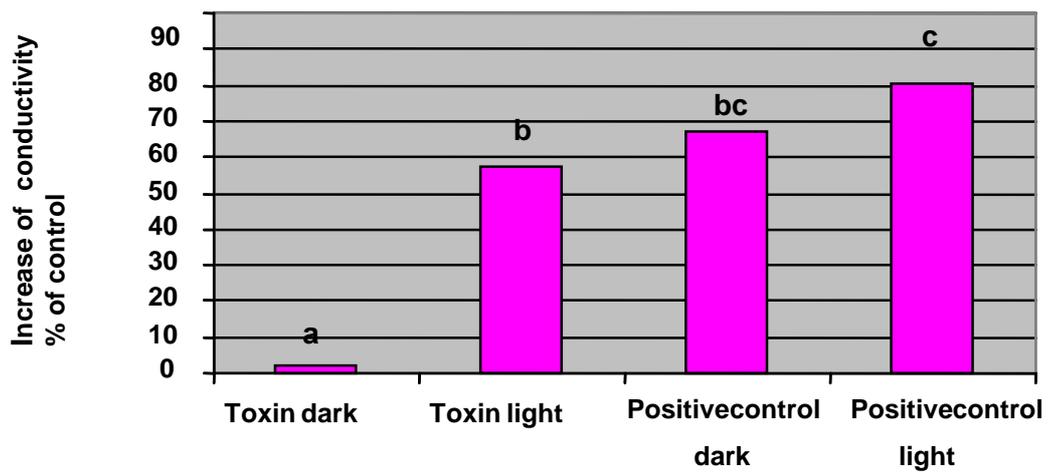


Fig. 53. Effect of alternethanoxin A (tested at the concentration 2 mg/ml) on electrolyte leakage in leaves of *Cirsium arvense* (18 h post application)

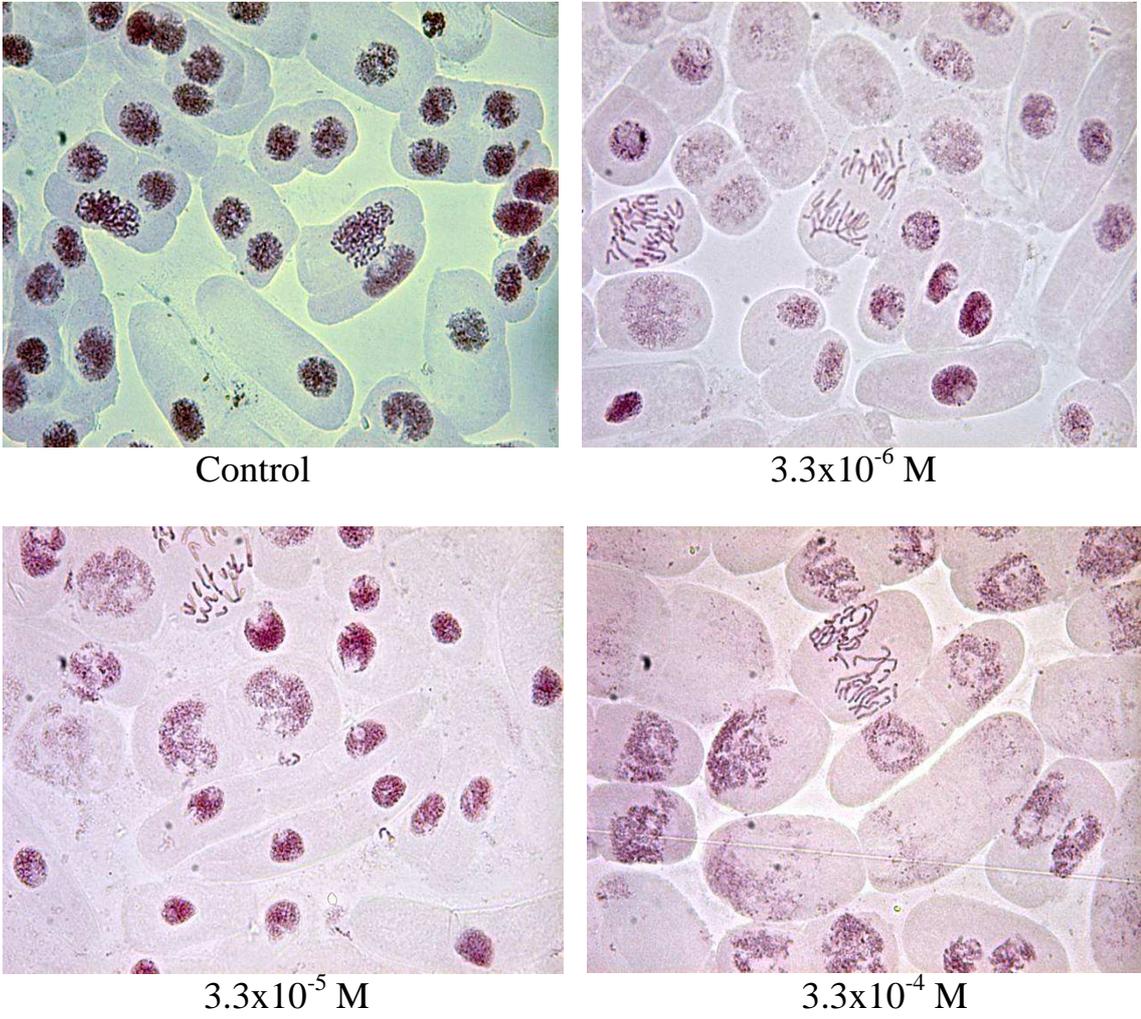
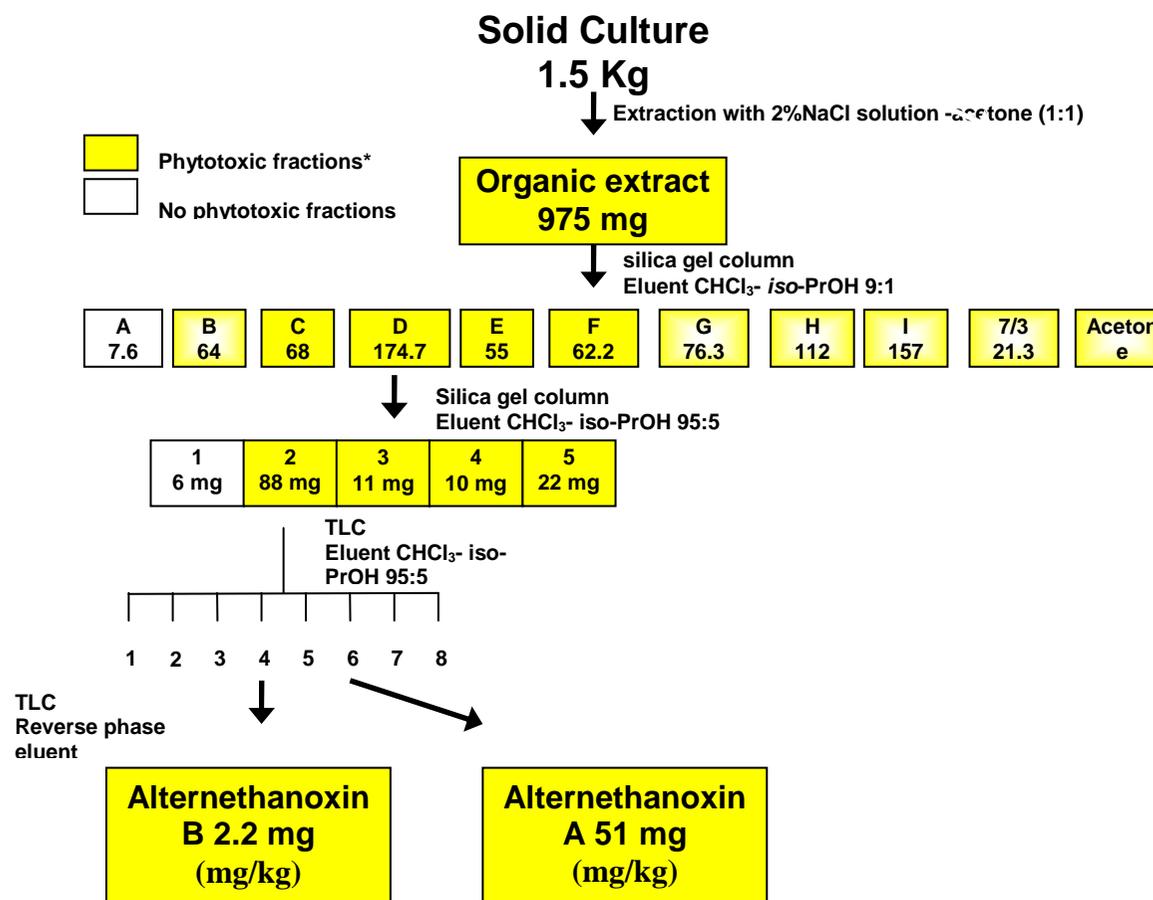


Fig. 54. Effect of alternethanoxin A on morphology of onion cells. 24 h after treatment.



Scheme 1. Process of extraction of solid culture of *A. sonchi*, and purification of the corresponding organic extract by column chromatography

*The phytotoxicity was assayed by leaf disc puncture assay on leaves of host plant

Table 1. Promising phytotoxins for use as natural herbicides

Microbial source	Phytotoxin	Target weed	Site of action
Fungi			
<i>Alternaria alternata</i>	Tenuazonic acid	<i>Datura innoxia</i>	
<i>A. alternata</i>	Tentoxin	Grasses, broad-leaved weeds	CF 1-ATPase
<i>A. alternata f. sp. lycopersici</i>	AAL-toxin	Garden cress	
<i>A. alternata f. sp. maculosa</i>	Maculosins	Spotted knapweed	
<i>A. zinniae</i>	Zinniol	Lettuce seedlings	Disruption of calcium-regulated cell processes
<i>Ascochyta hyalospora</i>	Ascochyte; hyalopyrone	Lambsquarters, prickly sida	Electrolyte leakage and inhibition of root growth
<i>A. caulina</i>	Trans-4-aminoproline	<i>Chenopodium rubrum</i>	Unknown
<i>Bipolaris cynodontis</i>	Bipolaroxin	Velvet leaf and pigweed	
<i>Cephalosporium spp</i>	1233A		HMG CoA synthase
<i>Cercospora kikuchii</i>	Cercosporin	Higher plants (not defined)	Lipid peroxidation and photo-sensitizing action
<i>Fusarium sp.</i>	Fusaric acid	Broad spectrum	Jimsonweed and duckweed
<i>Gliocladium virens</i>	Viridol		
<i>Helminthosporium sativum</i>	Prehelminthosporal	Johnsongrass	Unknown
<i>Irpex polyhedon</i>	Irpexil		Enzyme inhibitor
<i>Paecilomyces variotii</i> SANK 21086	Cornexistin	Dicotyledonous weeds and some monocotyledonous weeds	Aspartate amino transferase inhibition
Actinomycetes			
<i>Nocardia sp. no. 2–200</i>	Thiolactomycin	Higher plants (not defined)	Type II fatty acid synthetase
<i>Streptomyces hygroscopicus</i>	Hydantocidin	Broad spectrum	
<i>S. hygroscopicus</i>	Polyethrin A	Garden cress	Inhibits ceramide synthase
<i>S. hygroscopicus var. geldanus</i>	Geldanamycin	Garden cress	
<i>Streptomyces sp.</i>	Anisomycin	Barnyard grass and crabgrass	Inhibition of photosynthesis
<i>S. suganensis</i>	Herbicidins A/B	Monocotyledonous and dicotyledonous weeds	
<i>S. toyacaensis</i>	Toyocamycin		
<i>S. hygroscopicus</i>	Nigericin		Potassium ionophore and photophosphorylation inhibitor
<i>Streptomyces sp. A7847</i>	Herboxidiene	Broad spectrum	Unknown
<i>Streptomyces sp.</i>	SF-701	Barnyard grass	Inhibition via starch synthesis
<i>Streptomyces sp. 620061</i>	Pyrizadocidin	Giant foxtail	Electron transport inhibition
<i>Streptomyces sp. AM-3672</i>	Herbimycin	<i>Digitaria spp.</i> , giant foxtail (<i>Echinochloa crusgalli</i>), <i>Chenopodium</i> and <i>Portulaca</i>	Unknown
Bacteria			
<i>Pseudomonas syringae var. tabaci</i>	Tabtoxin	Broad spectrum	GS-GOGAT pathway
<i>P. syringae var. phaseolicola</i>	Phaseolotoxin	<i>Glycine wightii</i> and <i>Macrophillium atropurpureum</i>	Ornithine carbamoyl transferase inhibitor
<i>P. syringae pv. atropurpurea</i>	Coronatine		
Cyanobacteria			
<i>Scytonema hofmanni</i>	Cyanobacterin	<i>Lemna gibba</i>	Inhibits PSII site

Table 2. ^1H and ^{13}C NMR data of alternethanoxins A (24)^{a,b}

Compound	24		
Position	$\delta\text{C m}^c$	δH	HMBC
1	148.8 s		MeCO
2	128.6 s		
3	109.5 d	6.21 s	MeCO
4	160.0 s		H-3
5	160.0 s		
6	109.5 d	6.21 s	
6a	153.0 s		H-10, H-9
7	167.3 s		H-8, OMe
8	122.2 d	7.46 d ($J=7.5$ Hz)	H-10, H-9
9	131.0 d	7.34 dd ($J=7.5$ and 7.1 Hz)	H-8
10	121.4 d	7.11 d ($J=7.1$ Hz)	H-8
10a	130.4 s		H-9
10b	109.8 s		H-6 and/or H-3
MeO	52.5 q	3.62 s	
<u>MeCO</u>	198.7 s		H-3
<u>MeCO</u>	22.0 q	2.23 s	H-3

^aThe chemical shifts are in δ values (ppm) from TMS.

^b2D ^1H , ^1H (COSY) ^{13}C , ^1H (HSQC) NMR experiments delineated the correlations of all the protons and the corresponding carbons.

^cMultiplicities were assigned by DEPT spectra.

Table 3. ^1H and ^{13}C NMR data of alternethanoxins B (25)^{a, b}

Compound	25		
Position	$\delta\text{C m}^c$	δH	HMBC
1	148.8 s		MeCO
2	112.4 s		HOC-C(3), H-3, MeCO
3	107.2 d	6.73 s	HOC-C(3), MeCO
4	161.1 s		HOC-C(3)
5	155.6 s		H-3
6	111.4 d	6.62 s	
6a	150.8 s		H-8, H-9
7	169.2		H-8, OMe
8	125.4 d	7.36 d ($J=9.0$ Hz)	H-9
9	122.2 d	7.47 d ($J=9.0$ Hz)	H-8
10	152.2 s		
10a	155.6 s		H-9
10b	118.6 s		
MeO	53.2 q	4.00 s	
<u>MeCO</u>	180.1 s		H-3
<u>MeCO</u>	22.7 q	2.43 s	H-3

^aThe chemical shifts are in δ values (ppm) from TMS.

^b2D ^1H , ^1H (COSY) ^{13}C , ^1H (HSQC) NMR experiments delineated the correlations of all the protons and the corresponding carbons.

^cMultiplicities were assigned by DEPT spectra.

Table 4. Mitotic index of onion root tips exposed to alternethanoxin A

Concentration	Total cells observed, %			
	Prophase	Metaphase	Anaphase	Telophase
0.1 mg/ml	1.45	3.60	1.50	0.20
0.01 mg/ml	2	1.76	1.23	0.05
0.001 mg/ml	1.60	2.60	0.80	0.20
0	1.24	3.80	1.30	0.70