1 Introduction

A chemical communication system that can transmit information with high efficiency in daily life also exists in the animal and plant world. Organisms communicate entirely by exchanging chemical information, which is carried out by chemical messenger substances.
The investigation of this chemical language was assigned to the research field of “chemical ecology”. This research field deals with the chemical aspect of correlation inside ecosystems and allows the behavioural modifying natural substance with communication structures.

The International Society of Chemical Ecology (ISCE) provided a definition for the interdisciplinary research area of chemical ecology on their Internet-homepage:

> **Chemical ecology came to be recognised as a distinct interdisciplinary research area about three decades ago. It deals with the intriguing chemical mechanisms, which help control intra- and interspecific interactions among living beings. All organisms use chemical signals to transmit information; “chemical languages” are the oldest forms of communication. Research in the field of chemical ecology is concerned with the identification and synthesis of the substances which carry information, with the elucidation of receptor and transduction systems which recognize and pass on these “semiochemicals”, and with the developmental, behavioural, and ecological consequences of chemical signals.**

Within the research field of chemical ecology many scientists as chemists, biochemists and biologists are engaged in interdisciplinary studies.

Chemical communication between individuals of the same or different species is widespread and occurs throughout the animal kingdom. It appears to be mediated among the animals. An animal´s chemical communication system is usually assumed that it normally perceives the chemical signal under investigation and is best suited to act as the biological detector for the presence of active compounds.

A model for a typical communication system consists of three parts:

1. Pheromone emission from a signalling organism
2. Pheromone transmission through a medium (air/water)
3. Pheromone reception by the recipient's olfactory (smell) or gustatory (taste) organ.

Scientific investigation of chemical signals is a much more recent chemical ecological research. This investigation started in the nineteenth century with the experiments of naturalists who were intrigued by the ability of female moths and butterflies to attract the male. However, scientists only slowly achieved a satisfactory interpretation of these phenomenons (Agosta, 1992).

Since Butenandt (1959) who firstly investigated an intraspecific chemical signal as the sex attractant of insects intensive research has been performed on the isolation and elucidation of signalling compounds. Chemical ecology basically evolved from terrestrian species silkworm moth, *Bombyx mori*, however the marine habitat has been widely investigated throughout the last 20 years as well. Today, a large body of signalling compounds have been described both biologically and chemically.

Chemical messenger substances are widely used within most species of the animal kingdom, depending on the particular species involved. They serve to attract a mating partner or stimulate that partner to copulate, to alarm others to stay away from danger or to react by forming colony sites. There are also various other behavioural functions.

Chemical ecology comprises the study of those interactions of organisms that are mediated by the chemicals they produce. An important part of these interactions relates to odour communication in species (Ritter, 1978).

Many studies have shown, that marine organisms use chemical signals as well as terrestrial organisms and display response to this signal depending on the behavioural situation, the physiological state of the individual and other factors e.g. temperature, water, light, etc. controlled by the social and physical environment.
A study of chemical messenger substances involved in species communication usually requires a laboratory bioassay. This assay is based on the behavioural changes of the test organism in response to the test compound.
The isolation of an unknown biologically active compound from the complex substance mixture requires a bioassay. Ideally, a bioassay should be performed at every major step in the isolation of a natural mixture or secretion.
The difficulty of designing suitable bioassays under laboratory conditions complicates the test of the communicative role of smell (Mykytowycz, 1979).

1.1 Chemical messenger-substances-Pheromones

The new term of pheromone unlike hormones was found in 1959. The original name, which Bethe (1932) called “ectohormones”; was followed by some authors and rejected by others.

Therefore, P. Karlson and M. Lücher (1959) proposed this new term “pheromone” which is derived from Greek “pherein” meaning “to transfer” and “hormone” meaning “to excite”, “to stimulate” for this group of bio-active substances.

A pheromone is a chemical compound secreted from an animal, which elicits a behavioural or physiological response in another conspecific animal. In this fashion, a pheromone acts as a chemical messenger among individuals.
According to Schorey (1976), a pheromone can be defined as a chemical or mixture of chemicals that is released to the exterior by an organism of the same species, can be synthesised *de novo* in specialised glands in the producing animals.
In the practical world, an investigator who observes that one or more unknown chemicals released by an animal cause a behavioural response in another

animal of the same species call the chemical message a pheromone (Schorey, 1976).
In the beginning, the term pheromone was used for the designation of insect messenger substances, however it was also used for vertebrates and plants. Pheromones therefore comprise a special group of compounds among the variety of intraspecific messengers.

Two distinctive categories of chemical messenger substances in the animal kingdom have been defined:
a.) intraspecific (i.e. individuals of the same species) and
b.) interspecific (i.e. individuals of the different species).

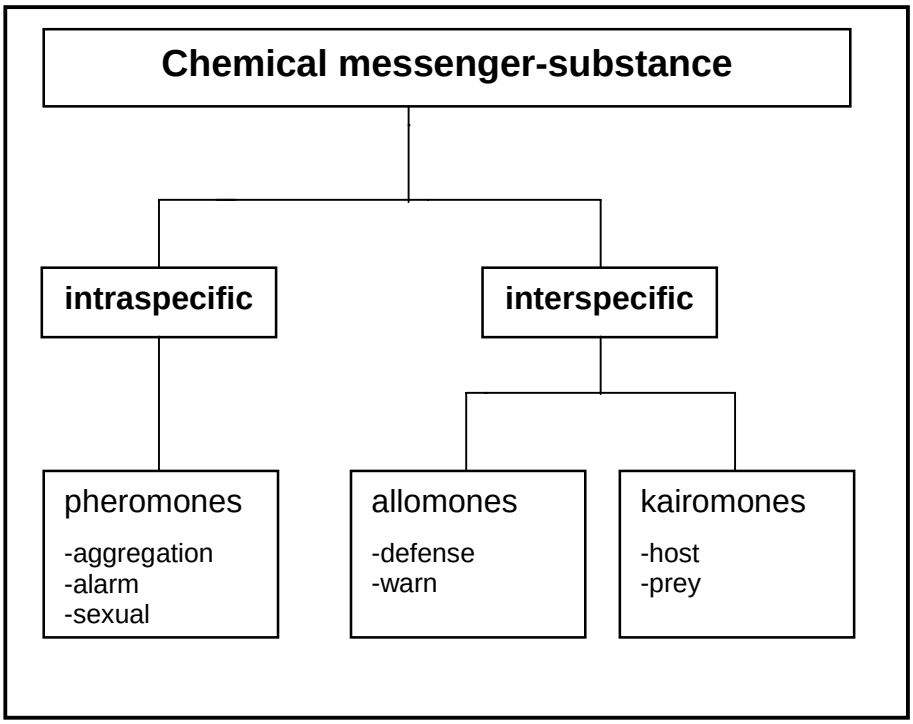


Figure 1.1. Categories to chemical messenger-substance

According to Brown et al. (1970) allomones are defined as chemical substances produced by an organism, which induce a behavioural response in case of a contact with an individual of a different species. These kinds of substances favour the emitter. For example, allomones comprise defensive and warning signals.
Allomones such as interspecific chemical defensive substances are pirated from the environment or other organisms, however used as pheromones. Perhaps the simplest form of ecological use of chemicals to convey messages between members of the same population is exemplified by honeybee work-

ers. In fact they imprint on the olfactory and taste characteristic of a plant and transmit this information to other workers (Thiessen, 1977).

In contrast to allomones, kairomones are interspecific messengers, which favour the recipient rather than the donor. For example, kairomones comprise prey and host fragrances. They include the substances that mediate the positive responses of predators to their prey, herbivores to their food plants, and parasites to their hosts (Brown et al., 1970).

The pheromonal culture can be divided into subcategories depending on the biological phenomena. For example, compounds that lead to aggregation, coordinate reproductive behaviour, elicit an alarm reaction or sign a trail.

Wilson (1963) distinguishes two types of pheromones, a “releaser pheromone” and “primer pheromone”. A releaser pheromone immediately elicits a behavioural response in the receiving animal’s nervous system. It stimulates specific chemosensory organs and relays the messages via their sensory nerves to the central nervous system.

Primer pheromones also relay messages to the central nervous system, however, without direct behavioural response.

The pheromone is said to have a primer effect, if it alters a set of long-term physiological conditions so that the recipient's behaviour can subsequently be influenced by specific accessory stimuli. On account of the practical importance in the natural environment, pheromones were firstly investigated in insects. Butenandt et al. (1959) elucidated the chemical structure of the sex pheromone from silkworm moth *Bombyx mori*, which was named accordingly as Bombykol (see figure 1.2).

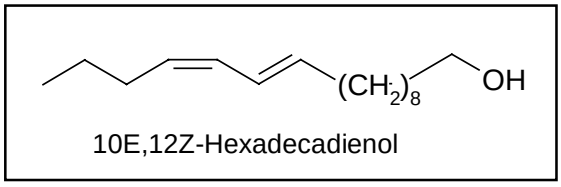


Figure 1.2. Structure of sex pheromone (bombykol) from *Bombyx mori*.

Sex pheromones constitute a large and important category of chemical ecological research. The function and meaning of many insect sex-attractants has been developed by chemical separation and identification of pheromones chemically. Since this successful investigation with bombykol the pheromone research is still continuing today.

During the past years, many scientists have elucidated a large body of (structure explanations on the field) terrestrial pheromones. However, only a few marine pheromones have been structurally identified. Because of this situation scientists' interest is directed to identifying and isolating the new structure of pheromones in the aquatic living species.

1.2 Pheromones in marine organisms

Although there is a great number of behavioural observations, which indicate an influence of pheromones in marine organisms, the chemical structure elucidation of the corresponding substances has been described successfully in only a few cases.

According to Agosta (1992), insect pheromones are convenient for chemical studies in other ways. In general, the compounds employed as signals by insects are chemically simple, and thus it has been relatively easy to determine their structures and then synthesised them. Moreover, the technical difficulties of monitoring of marine organisms are more than for those of terrestrial insects.

Therefore, most of the study of pheromone chemicals involved in insect communication (with a development of a sensitive bioassay) is easier than that of marine communication. Krittredge et al. (1971) firstly postulated that the sex pheromone of female crab *Pachygrapsus crassipes* might be the crustecdysone and this investigation is the beginning of the marine invertebrate research.

1.2.1 Molluscs

The strategies of chemical defence in various marine molluscs are well known. They accumulate secondary metabolites in their digestive gland through the food chain and produce defensive compounds. The structures of new compounds from the coloured sea slug *Navanax inermis* were investigated by Fenical et al. (1979). They reported that a bright yellow secretion produced by *N. inermis* from glandular tissue as an intraspecific alarm pheromone, which expels a migrating *N. inermis* population away from areas of high predator population. This classical yellow alarm pheromone was readily chemically

identified. There are three major components; navenones A, B, and C (see figure 1.3) which are chemically light sensitive. The system of double bonds present in the navenones causes them to be bright yellow which turns out to be biologically significant (Fenical et al., 1980).

The alarm pheromone of *N. inermis* in not readily diluted or washed away by seawater but remains suspended and protected in the slime track on the bottom. Without degradation through the action of sunlight, it might remain active for days. Sunshine obliterates the yellow warning message after a reasonable length of time.

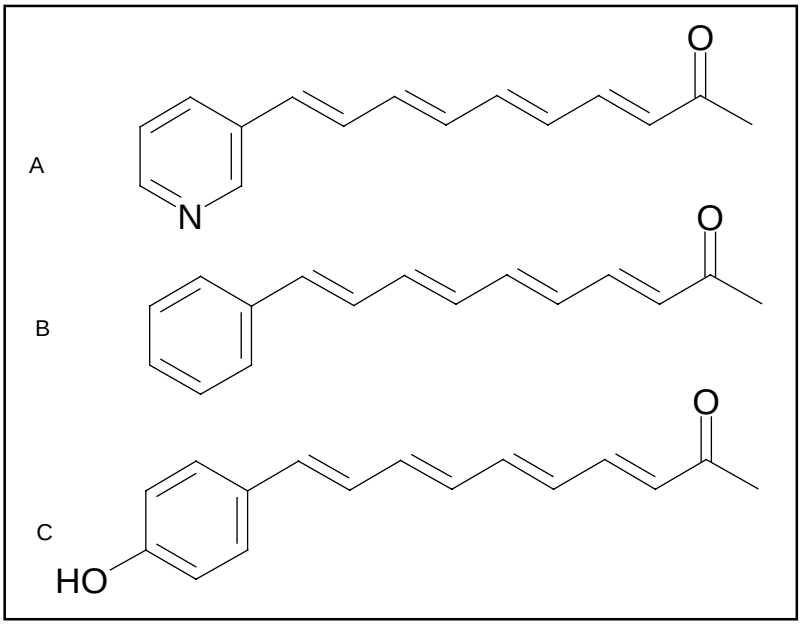


Figure 1.3. Navenones A, B, and C (from Fenical et al., 1980)

1.2.2 Sea anemone

Howe and Sheikh (1975) identified the chemical structure of an alarm pheromone in the sea anemone *Anthopleura elegantissima* which responds with characteristic contraction to a pheromone released by other members of the species. It is one of the chemically best investigated invertebrate pheromones and is postulated as the second pheromone from a marine invertebrate species to be isolated and fully characterised chemically.

Anthopleura elegantissima is a common sea anemone about 2 centimeters in diameter that decorates intertidal rocks along the California coast. If it is

wounded, this animal produces an alarm pheromone that warns neighbouring members of its species. On receiving this signal, an anemone undergoes a rapid convulsion unlike that produced by any other stimulus. First it gives a series of quick, convulsive flexures of its tentacles withdrawing then into the mouth cavity, and the mouth closes tightly. All of this happens in less than 3 seconds. If the alarm signal does not persist, the anemone resumes normal behaviour within 2 hours. This alarm substance called anthopleurine (see figure 1.4) was clearly postulated and analysed on the basis of chromatographic and spectrometric methods. This pheromone is an ionic compound. The positive ion interacts with the receptor molecule in the recipient anemone.

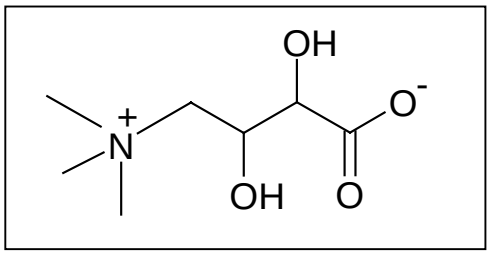


Figure 1.4. Structure of anthopleurine

Fortunately ecologically derived chemicals could be used as alarm signals, donators of colony sites, identification cues for species membership, notification signals for movement through various environments, and indicators of the kinds of substrates being ingested and metabolised.

These chemicals must have optimal transmission, qualities, be readily detected by conspecifics or enemies and reflect important biological or ecological states. Although for a long time pheromones determination of invertebrates in general was supposed to be much simpler, sexual and alarm pheromones produced by marine vertebrates especially fish have already been studied, but not intensively. Therefore, concerning the pheromones of fish would be considered as follows.

1.3 Pheromones of fish

Most fishes have a bony skeleton and are grouped together as teleoster. The vast majority of the world´s river and lake fishes belong to a group of b

ony fishes called Ostariophysi which comprises more then 5000 species (Agosta, 1992).

Living in an aquatic environment often devoid of light but rich in dissolved compounds, fish have evolved highly developed chemosensory and chemical signalling systems. These systems have a variety of functions of fish behaviour including schooling, individual recognition, recognition of predators, orientation, aggression, the promotion of reproductive synchrony, homing, crowding-influenced depression of viability and alarming conspecific (Pfeiffer, 1982).

In all these aspects of behaviour, the most of them concerning the chemistry of the presumed signal substance and response are as yet unclear. However, it is well known that fish have established communication, the prime necessity for social life through their chemical senses, olfaction and taste. Since communication between individuals of a species of fish by chemical agents was first demonstrated in 1932, such a process has been suggested in many aspects of fish behaviour and development (Solomon, 1977). Afterwards, one of classic observations of the chemical communication between fish was made by von Frisch (1938, 1941a, b) who detected the fright reaction in European minnow *Phoxinus phoxinus* to the order of a physically damaged conspecific.

It is difficult to draw clear chemical studies about alarm pheromone and especially fish pheromone systems are poorly understood.

Our understanding of even the best-known pheromone system among the fish is far from being complete.

The elucidation of chemical structure associated with fish in their environment is very little known and we are particularly interested therefore to identify these alarm substances structurally. When considering the structure elucidation it would be necessary to mention the history of alarm substance.

1.3.1 Alarm substances of fish

Von Frisch (1941b) initially observed and then answered this question and defined the term “alarm substance” (Schreckstoff) as a substance produced and perceived, intraspecifically, for the communication of danger, and signals “danger-get out of here”.

These substances are currently defined as “alarm pheromone”, according to the definition of “pheromone”, encompassing all intraspecies chemical communication (Karlson and Butenandt, 1959; Kittredge et al., 1974, and Fenical et al., 1979).

The alarm substance which is detected only by the sense of smell, the sense of taste is not involved is the pheromone and is the fish pheromone known with certainty (Pfeiffer, 1977). It was universally considered as Schreckstoff of fish.

Pfeiffer (1960) has suggested the name Schreckstoffzellen “alarm substance cells”. The epidermal cells have been localised as responsible for the alarm substance. He reported that teleost fish skin shows two types of epidermal secretary cells: the mucus cells (Becherzellen) and the club cells (Kolbenzellen). The former open onto the surface of the skin and pour out their mucus as from a beaker; the latter vary in distribution and appearances in different kinds of fish.

The club cells are not connected to the surface. Injury of the skin releases the contents of these cells and only in this way does the alarm substance reach the surface. (Pfeiffer, 1977).

Comparative studies based on the examination of alarm substance cells may provide information about which cells are present in most species of Ostariophysi. In some species it differs in structure.

Bardach has also provided Pfeiffer that Schreckstoff occurring in the club cells of Osteriophysean skin is a soluble substance or mixture of substances of unknown nature, but is likely to be of small size and perceptible smell (Bardach and Villars, 1974).

Epidermis with alarm substance cells in several species have been reported. There has also been progress in detailed research and much has been published about the structural features of the alarm substance cells during the last thirty years. In fact, among of the alarm substance cells of the fish, which have been reported previously, are still being studied intensively and extensively by the two groups of scientists: Pfeiffer and Smith.

Smith (1989, 1992) discussed that the alarm system is based on specialized epidermal club cells, alarm substance cells that release effective amounts of the alarm substance only when they are mechanically damaged.

Peters and co-workers (reference cited in Smith, 1992) examined the fine structure of the alarm substance cells of the Characinidae species using transmission electron microscopy and found that the alarm substance cells of these species did not differ from each other and were essentially similar to those of other ostariophysan.

Alarm pheromones in fish have been described for several orders but details are only known for the Ostariophysi and the Gonorhynchiformes.

Finally, Pfeiffer concluded that the alarm substance cells were found histologically in all species which contain the alarm substance according to the behaviour experiment (Pfeiffer, 1974).

The alarm substance cells (see figure 1.5) of European minnow are located in the outer layer of the skin, where they can be ruptured easily upon injury. The mucus cells discharge the slimy substance that forms the protective outer coating of fish (Agosta, 1992).

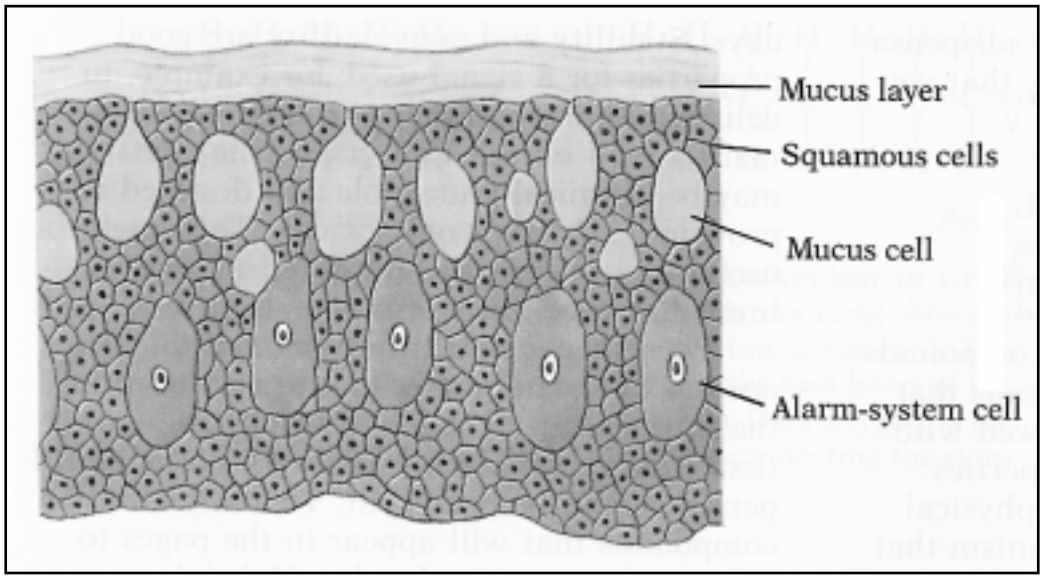


Figure 1.5. The alarm substance cell of minnow (from Agosta, 1992)

When an attracting predator damages the skin of an ostariophysi fish, club cells in the epidermis containing an alarm pheromone or Schreckstoff are broken and the alarm pheromone is released into the enviroment. Nearby fish respond to this waterborne alarm substance with a fright reaction.
The ability to produce and respond to an alarm pheromone is not present on hatching. Pfeiffer (1974) reported that the alarm reaction appears in minnows at about 50 days, the substance being present in the skin for a week before this. The shoaling habit develops at about 25 days.

1.3.2 Olfactory detection

From the second half of the 19th century scientists have studied the morphology of the olfactory organ in fish which demonstrates a great variety in its morphological characteristics. It plays an important role to determine their degree of behaviour.
As mentioned above, the alarm substance is detected only by sense of smell; the sense of taste is not involved. Von Frisch (1941b) proved this investigation as follows.
He removed the Nervus olfactorius and Bulbus olfactorius in 10 schools of *Phoxinus* and kept the minnows in isolation until they recovered. The removal of the olfactory bulb prevented regeneration of the olfactory organ. Operated fish subsequently did not respond to alarm substance (Pfeiffer, 1974).
Agosta (1992) gives further explanations to von Frisch´s (1941b) finding how European minnows detect alarm pheromone through their sense of smell. As the minnow swims, a ridge of skin on each side of its head direct water through the opening known as the enterior naris and into the olfactory chamber. After passing over the olfactory cells, the water leaves through the posterior naris (see figure 1.6).

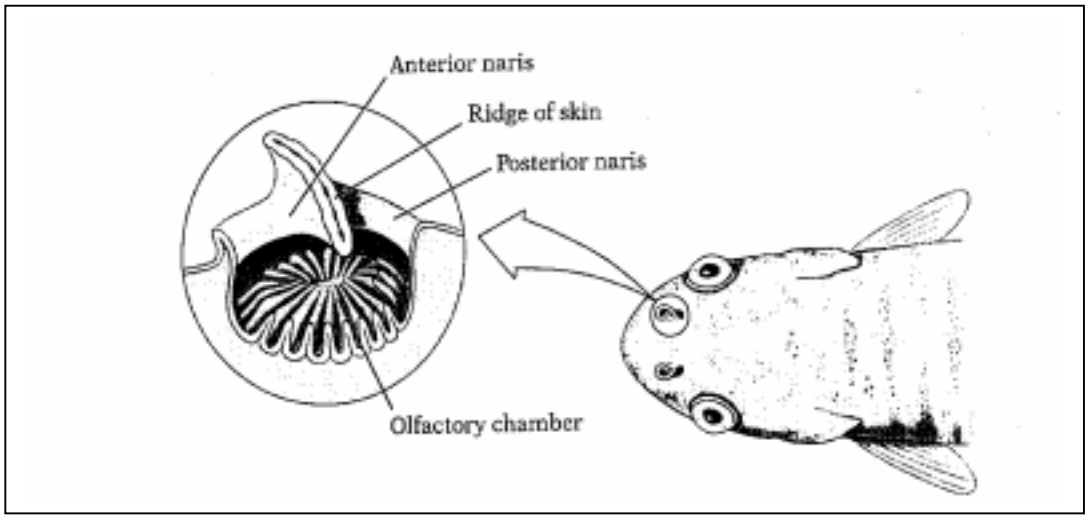


Figure 1.6. The structure of olfactory chamber of minnow (from Agosta, 1992)

Accompanying the alarm substance cells the important point is the biological fright reaction called “alarm reaction”.

1.3.3 Fright reactions

Von Frisch (1938, 1941b) first detected fright reaction and indicated that the Schreckstoff elicits a fright reaction in conspecifics causing them to move away from an area of danger. He intensively studied and confirmed his pioneering observation with schooling cyprinids of the order Ostariophysi by field and laboratory experiments (von Frisch, 1941b).
Fish assembled at a feeding tray seem to be terrified when the substance is introduced and flee a short distance in confusion. Then they crowd together and retreat. In the aquarium experiments, the fright reaction gives similar observation as field experiments do (Pfeiffer, 1977).
Fifty years of investigation has yielded a large body of information about behavioural and physiological aspects of the ostariophysian fright reaction.

Depending on the behavioural features of several species some of fright reactions may be similar to each other, but some may vary according to their way of life and habitat. Here will be given the examples:

Phase 1: The tench, *Tinca tinca* (L.) may swim excitedly with their heads against the bottom and their bodies at an angle of about 60° to the floor (Schutz, 1956);

Phase 2: the gudgeon, *Gobio gobio* (L.) become motionless and show no movement for several minutes (Schutz, 1965; Pfeiffer, 1963b);

Phase 3: the hornhead chub, *Hybopsis biguttata Kirtland* sink to the bottom and start spitting gas for a considerable time (Verheijen, 1963);

Phase 4: the genus Esomus (flying barbs) flee to the surface when they are alarmed, crowd together there and swim hastily, frequently jumping out of the water (Schutz, 1956) and

Phase 5: the hatchetfish flee towards the depth where they form a dense school when alarm substance is introduced (Pfeiffer, 1963c).

These characteristic strong reactions were only produced from injured skin (von Frisch, 1941b). It was already known that minnows are alarmed by a great variety of stimuli and that best results of the fright reaction can be received in the initial placing of fish in an aquarium. Von Frisch confirmed repeatedly alarm reactions with minnows in field and laboratory experiments. Both of these experiments have shown similar fright reactions with different intensities.

It was postulated that many fright reactions are biologically significant and many of these papers have been reviewed by Pfeiffer (1962, 1963a, b, c, 1966, 1974, 1977, 1982) and Smith (1977, 1992). It must be very important to determine and confirm alarm reaction definitely to prove the chemical nature of alarm substance in aquatic animals.
Pfeiffer (1977) suggested that fright reaction has probably contributed markedly to the biological success of the Ostarioiphysi, the group that constitutes the majority of fresh-water fishes and consists of some 5,000 to 6,000 known species.

1.4 The chemistry of pheromones

Pheromones are generally single chemical compounds or mixtures of chemical compounds. Many contain such functional groups as carbon-carbon double bonds, carbonyl, hydroxyl, carboxyl, ester and amino groups.
As described above marine pheromones are so long found as small molecules, polar, hydrophile oligopeptides, as lipophile, volatile, unsaturated hydrocarbons; as polar, non-volatile, water-soluble, amino acid derivatives and purine derivatives.
Due to the wide variety of pheromone structure, it is certainly difficult to show which substance contributes to which related pheromone.
Most known pheromones contain 5 to 17 carbon atoms. This range allows for sufficient chemical combinations and molecular complexity to create a molecule that is unique to a given species.
Of course, the chemical composition and physical properties of a pheromone relate to its role as a messenger.

Hüttel (1941) gave a first proposed on the chemistry of the alarm pheromone and suggested that minnow alarm substance was a purine-or pterin-like compound, was nonvolatile and extremely water-soluble. Unfortunately, after his finding, relatively very little progress has been made in the identification of alarm pheromones in fish. Reutter and Pfeiffer (1973) investigated a suggestion that the alarm substance cells from minnows contain flourescent pterins. Tucker and Suzuki (1972) have studied the biologically stimulating effect of the skin extracts from white catfish and suggested that the alarm substance was a mixture of several compounds, including some amino acids and oligopeptides. Reed et al. (1972) interpreted that a ring or double ring compound might be involved in alarm substance. In regard of the alarm substance Ruddy and Baeder (1973) concluded that it was a histamine or a histamine-like substance. In an effort to isolate and identify the alarm substance of minnows, Pfeiffer and Lemke (1973) tested skin extracts on the behaviour of schools of the giant danio and concluded that the alarm substance of cyprinids was probably a pterin like ichthyopterin. They were also able to demonstrate the fright reaction to isoxanthopterin. Pfeiffer (1978) investigated again the bioassays with 59 pteridine, purine and pyrimidine derivatives. Three of the pteridine derivatives (2,6-diamino-4-oxodihydropteridine, isoxanthopterin and 6-

acetonyl-isoxanthopterin) tested elicited the fright reaction. Purine and pyrimidine derivatives tested were ineffective. From these experiments, it was found a strong activity of isoxanthopterin and its derivatives. But there were still doubt, whether these active pterins were identical with the natural alarm pheromones (Pfeiffer,1975).

Although alarm substances and alarm reactions of many fish have been biologically studied and published by several authors, their related chemical structure had been unknown until the work of Argentini (1976). He isolated Schreckstoff from skin extract of the minnow *Phoxinus phoxinus* and found that alarm substance is probably hypoxanthine-(3N)-oxide (see figure 1.7). He isolated the active fraction chromatographically on cellulose with *n*-butanol/acetic acid/water (20:3:7) as solvents controlled by R_f values. Argentini characterised the alarm substance as a colorless, non- flourescent substance, poor soluble in water, it shows a purine similar UV-spectra and is stable in water only for 26 hours.

He assumed that the peak m/z 152 in mass spectra is the molecular peak of N-oxide of hypoxanthine and produced synthetically hypoxanthine-(1N)-oxide and hypoxanthine-(3N)-oxide.

In his work, TLC at cellose showed the same R_f value for the natural product and hypoxanthine-(3N)-oxide. Finally it was found biological activity only for hypoxanthine-(3N)-oxide. A detailed chemical analysis of the purified pheromone was not provided by him.

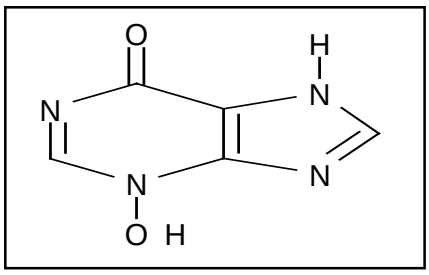


Figure 1.7. Structure of hypoxanthine-(3N)-oxide

Indeed after his chemical finding, the research on this topic concerning structural identification of alarm pheromone in fresh water fish was never done again by chemists who are otherwise active in pheromone research. Here is therefore a new area of alarm pheromone research with fish.

1.5 Objective

Pheromones are extremely widespread in nature, and chemists have studied the pheromones of insects and a few aquatic organisms. However, final proof of the chemical structure of the naturally occurring alarm pheromone in fish is not adduced up to now.

The aim of this research is to present considerable evidence for the existence of an alarm pheromone in the super order Ostariophysi by using the isolation, identification, and laboratory bioassays.

Emphasis is made on the fish *Danio malabaricus*, order Ostariophysi, which is the subject of study in this research. *Danio malabaricus* is proposed because it presents the best-understood function of alarm reaction.

2 Material and Methods

2.1 A prerequisite of the pheromone isolation and identification

The approach to alarm pheromones by actually separating and isolating the biologically acting chemical component from a complex substance mixture requires to test organisms that react towards the isolated substance.

A bioassay is required which guarantees a reproducible response if possible under natural conditions. The basic assumption for bioassays in the category “fright reaction” is described previously. An essential prerequisite is the consecutive supply of organisms in order to be able to extract pheromone-containing sample as well as to carry out bioassay of fish.

In case of alarm pheromone isolation and identification it would be an important step to focus on the behaviour of the species giant danio, *Danio malabaricus.*

2.2 Criteria for method selection

According to the following criteria considered in the method selection:

- On account of the unknown chemical stability of the pheromone possible suitable methods should be carefully used.
- There should be no chemical changes of the messenger-substances that might limit or suppress evolution of the bioassay.

- All chemicals (solvents, elution solvent) added to biologically active initial probes, should be easily, quickly and completely removable. Only chemicals with high degree of purity shall be used.
- No irreversible action should occur from chemicals with the biologically active component.
- Methods must guarantee the accumulation of a large amount of messenger substances for instrumental analytical investigation.
- Methods must be carried out in reproducible form.

From the final optimised methods a purity criterion should be derivable.

2.3 Origin of the Fish

The majority of the Worlds River and Lake fish belong to a group of bony fish. Most of the fish are familiar freshwater species. It is important, when selecting the fish for alarm pheromone research, to keep in detail about investigated fish.

2.3.1 Giant Danio, *Danio malabaricus* (Jerdon 1849) (Cyprinidae, Ostariophysi, Pisces)

Many shoals of fast swimming species from the Carp family exist in clean current water on the Western Coasts front India and Sri Lanka (Ceylon). For example, *Danio malabaricus* also belongs to this family. It is an omnivorous fish and therefore feeds on mixed food (Petrovicky, 1980).

They can grow up to 15 centimetres length in their natural enviroment. However, they only grow to the size of 10 centimetres length in the aquarium. The temperature of fresh water is about 25 to 28°C. In the natural setting, their colour is steel blue along the back, with irregular golden bright lines on anterior half, four or five bands along the sides, two central one coalescing to form a broad bluish band along the middle of the caudal fin. This makes the fish easy to recognise (Jayaram, 1991).

2.3.2 Subjects of fish

The subjects were adult specimen of both sexes, 3-5 cm in length, obtained from local suppliers (Pelz Aquaristik, Bondorf, Germany). In general, in most of the bioassays the same sizes and fish ages were selected.
They were fed with tetramin dried food once or twice daily and were maintained on a 12-h light: 12-h dark cycle. Water temperature in the aquariums was adjusted to maintain at 25°C. Covered glass tanks were used in all the experiments. Tanks were covered with black papers to avoid visual contact between fish in different tanks. Each tank contained its wad filter (Hagen) and the fish were placed in groups of 7 to 10 in an aquarium containing 20 liters of water (40×25×25) cm and a bubbling air stone. The flow rate of freshwater was adjusted to approximately 200-300 $mL \cdot min^{-1}$.

Danios fish were maintained in the laboratory at least 3 weeks prior to testing.
A weekly water alternation of an optimal fish tank must be carried out from at least 30% to 50 %.
Normally, the qualities of aquarium water are influenced through the kind and amount of the dissolved chemical substances.
Therefore it is noticed that fish-tank water must be observed and examined regularly for some factors. For example, pH-value, nitrite and nitrate concentration, ammonium concentration, copper content and carbonate hardness (KH) that were measured monthly to control the water in the aquarium. Aquarium water is neutral (pH-7 to 8,5).

2.4 Bioassays to evaluate the fright reaction

Previous investigations have shown that when the skin of an ostariophysian fish is damaged, alarm substance cells are broken and the alarm substances are released. Nearby conspecifics smell the alarm substance and show a fright reaction appropriate to their species.
Therefore bioassay plays the major role in the alarm pheromone isolation and identification, since it is the only way to prove the presence or absence of a biologically active substance in the complex sample composition.

Qualitative bioassays have been mostly required in alarm pheromone research to detect the presence of biologically active material and to determine the responses.

Every major separation step must be accompanied by bioassay and checked for its effectiveness. Also the fright reaction serves the pheromone as a visual signal spreading the alarm more rapidly through the social group.

The following factors must be set up to the bioassay,

- The bioassay must allow clear evidence on the presence of biologically active substance.
- The test must be repeatedly reviewed.
- The bioassay must allow a chemical stimuli from the tested species.
- The bioassay proceeding must approve the qualitative evidence on the amount of pheromone produced.

In the investigation of bioassay of fright reaction, the research was done with the schools of giant danio, *Danio malabaricus*, the species that was the focus of von Frisch´s original work (von Frisch, 1938, 1941b), Pfeiffer´s bioassay experiences (Pfeiffer, 1978) and Schutz´s distribution of fright reaction (Schutz, 1956).
Von Frisch (1941b) noted that the skin of poorly fed fish contained reduced amounts of alarm substance and that unhealthy fish show lower responsiveness. According to his records the feeding and health of the fish were controlled, and only daily healthy fish were tested.
Fish behaviour was recorded approximately 1 m in front of the test aquarium for viewing and filming. Each test was videotaped using a black VHS video camera.
The recording of each bioassay was measured on the monitor of a video recorder. From this video sequence the contribution of fright reaction in school danios was determined whether the test fish showed a positive reaction correctly or not.

The basic procedure followed in all tests was very similar to that used in the study of fright reactions in giant danio fish.

The laboratory bioassay was carried out during the morning after leaving the fish undisturbed for one to two weeks.
The fish were conditioned to the experimental aquarium prior to testing until they no longer showed fright reaction when a person approached the tank but remained near the feeding corner at the surface in expectation of food. The time needed for conditioning was usually about one or two weeks.
The danio fish soon learned about the source of food and were sufficiently trained for bioassay. Since fish may rapidly adapt to substances that elicit the fright reaction, most schools were tested only once with an effective substance. If the fish were tested repeatedly, a minimum time interval of two weeks elapsed between two successive experiments. After each bioassay with a fright reaction the aquarium was carefully cleaned and filled with aged tap water that was stored at 25°C (Pfeiffer, 1978).

2.4.1 Qualitative behavioural biological test

Undisturbed fish were recorded by obtaining a 20-min preview videotape at first for their normal behaviour. Thereafter, videotaping continued for the subsequent 30-min test period.
After their food had been introduced into the test tank, a test substance was then poured into the corner of their source-feeding place at the water surface quickly at intervals of 5-20 seconds. This process was done under video observation. The stimuli alarm reaction occurred with the following different substances in all experiments.

- Dilution of natural alarm pheromone only derived from skin extract
- Every chromatographic fraction from HPLC
- Dilution of different expected authentic substances

This substance consisted either of a natural alarm pheromone only derived from skin extract or every chromatograph fractions from HPLC and also other artificial chemical compounds (e.g. expected synthetic compounds).
Effective test substances should induce a fright reaction within 3-30 sec.
According to Pfeiffer (1978) fish which had assembled at the feeding corner close to the water surface seemed frightened and fled towards the bottom in

confusion; they then crowded together and retreated. Confidence returned after variable intervals ranging from minutes to several hours.

When test substances were given to an aquarium in the experiments, the variety of intensity of the fright reaction was investigated and the following five different situations were distinguished. Such stages were evaluated arbitrarily, and ranged from the most intense reaction where all fish were suddenly frightened and hastily fled, to a less obvious intimidation.

Reaction 1: (++)

The most intense reaction is sudden fright and rapid swimming towards the bottom. The fish may rapidly swim around the tank or stay motionless close together to the bottom, avoiding the feeding place for a long time.

Reaction 2: (+)

Clearly frightened; the school retreats to the bottom of the aquarium but slows down within 5-10 min and then approaches the middle of the water surface. Sometimes they may crowd the feeding area more or less confidently.

Reaction 3: (+-)

Only slightly frightened with somewhat confused behaviour; swimming to the middle of the aquarium where the school may crowd, but not retreating to the bottom and usually slowing down rapidly. Sometimes intimidated and less confident at the feeding corner with some crowding together, but the reaction soon disappears.

Reaction 4: (-)

Slightly uneasy with some crowding together in the area of the feeding place, but the reaction is short and they never leave the feeding area.

Reaction 5: (- -)

No reaction.

These categories permit useful quantitative work of giant danio fish and have been used in a similar way by von Frisch (1941b) and Pfeiffer (1963b, c).

The intense fright reaction began almost immediately within 3-5 seconds after the substance had been introduced into the test aquarium. In this case it became evident that these responses were demonstrated by the positive reaction (1).
Based upon the variety of test samples these behavioural reactions relate closely to danio fish.

The strong schooling tendencies, especially in unknown fish with no experience play a role in the response to the fright reaction, becoming more understandable with the demonstration of that reaction. With dried food, the fish were active with eating, exploring and social behaviour.

In general, diluted samples were injected for the bioassays and every positive test was characterised qualitatively by the delivered sample volume. The intensity of the fright reaction could be thought of as the product of the amount of test sample and the responsiveness of fish. By this measurement, it was possible to pursue or determine the distribution of biologically active substance. The same amount of separated fractions of identical concentrations from different large test volumes was required in tests quantifying the alarm behaviour. The quality of chromatographic separation could be proved as well by this parameter.

To assess the effects of pheromone activity and to observe the fright or alarm reaction more carefully with a digital camera (model: Agfa e Photo 1680), 5 ml of pheromone solution was prepared by scraping the skin of one freshly killed danio fish in 10 mL of Millipore water.

At present, every sequence of the test fish behaviour were photographed with the help of a digital camera. Reference to printed photographs of fright reaction is shown in figure 2.1. The behavioural reaction is recorded using the qualitative scale mentioned previously.

Figure 2.1a describes the normal behaviour of the fish.
Figure 2.1b describes the function of feeding after giving the food.
Figure 2.1c indicates the intense fright reaction after introducing the alarm substance within 10 seconds intervals of feeding.

Figure 2.1d represents the behaviour of the fish tested which clearly display fright response with the positive reaction 1 (++).

Figure 2.1a. The normal behaviour of the fish

Figure 2.1b. Feeding

Figure 2.1c. Introducing the alarm substance after 10 seconds intervals of feeding

Figure 2.1d. Fright reaction

As described in figure 2.1a-2.1d, the positive result is obtained by the alarm substance extracted from the fish skin.

To determine the qualitative bioassays of fright reactions:

- The amount of extracted material required to eliciting the various degrees of response.
- Activity of synthetic pheromone substances.
- The response of the fish to other relative substances must be known.

The result of bioassay can readily be qualified depending on the amount of pheromone. The intensity of the response became greater with increasing pheromonal amount.
All bioassays in this work were detected the alarm reactions with the parameters which are basically described above.
Of greater value is the qualitative bioassay, because it gives some measure of the amounts of active material present in a sample.
Moreover, the efficiency of alarm substance was also observed in the following experiments:
Schools of the unaffected fish were used and 5 mL of the diluted skin extract, which was previously prepared was firstly stored for 68 hours at room temperature. Afterwards, this aged sample was selected for bioassay. The behaviour of the fish was observed by using video camera. Dealing with the video result, aquarium danios responded to this aged skin extract and elicited the fright reaction as positive reaction no.1.
The diluted skin extracts from danio fish heating about 50°C for 30 minutes did not reduce its effectiveness to the fright reaction compared to the skin extract without heating.

2.5 Preparation of pheromone-containing sample

The important concern is to ensure preparation of the maximum amount of active material. The extraction of the skin of fish must be required at the beginning of the development of the separation procedure.
Donor danios fish were killed by a blow on the head and then a skin fillet from both sides of each fish was removed.

For preparation of alarm substance solution the following methods were applied.

[**1**] The skin was cut approximately 25 times on each side with a scalpel, scaled and then placed in Millipore water and shaken vigorously for 10 min. Then all diluted solutions were strained through a filter paper (No. 595, Schleicher & Schuell) to remove large particles. The supernatant solution from filtration was either used immediately or frozen for later testing.

[**2**] The skin of donor fish was macerated and placed in Millipore water and heated at 60°C for one hour. This procedure was performed under red light or in darkness. The liquid phase was obtained through a filtration.

[**3**] The skin was ground up in a mortar, homogenised [2500 rpm] (Bühler-homogeniser H-04), diluted with Millipore water and then stirred for 20 min. The combined homogenates were centrifuged by Hettich centrifuge (EBA III) to remove any solid particles. Otherwise, liquid supernatant was separated by centrifugation. By repeated extraction of the precipitate with millipore water, a complete separation of the messenger substance was achieved from the solid homogenous constituent.

[**4**] The skin samples were homogenised by a polytron homogenizer [2500 rpm] (Bühler H-04), placed in millipore water and stirred occasionally for 10 min. The homogenate was extracted with diluted 10% acetic acid for one day and filtered. The residue from filtration was extracted two more times with a diluted 5% acetic acid solvent as described above. After each extraction the samples were separated directly by centrifugation (Minifuge GC, Heraeus Christ) so that complete separation of alarm substance with the diluted acetic acid was obtained.

All of the aliquots extracted were stored at approximately –25°C until required. According to von Frisch (1941b), fish skin retains its ability to elicit fright reactions when held in water for 3 days at 16°C.
The method [1] was characterised to represent the alarm substance solution in its native composition. This process is similar to that used by von Frisch (1941b). The effectiveness of dark period from method [2] could be distin-

guished to prevent the light sensitivity of alarm substance cells. By the vacuum filtration an aqueous phase was separated from the protein phase. In contrast to method [1], it was possible by method [2], to achieve qualitative pheromone separation from the skin extract.

Method [3] and [4] were very similar. It should be noted that if the extracted alarm substance was soluble in water method [3] should be used. Otherwise if the investigated component was difficult to isolate in a large amount by water extraction, then method [4] should be used.
In methods [3] and [4] intensive mixture occurred with the small cellular materials. Comparative studies show evidence that pheromone containing sample which was extracted by method [4] was not only more complex but yielded a larger amount than those extracted by method [1], [2] and [3]. With the usage of method [4] it was considered that in the process of homogenisation the enzyme system can be freed. In this condition it might be intended to derivatisate the signal substance chemically and to decompose it biologically.

2.6 Sample storage

To store the sample it was placed in the deep-freezer at –25°C and was kept under Argon. Depending on the sample volume, glass bottles with screwcap (bulkhead), glass test tubes or reaction-vessels from plastic material (Eppendorf) were used. The vessels were closed with the lid or parafilm. This processing prevented a potential oxidation of the sample components by atmospheric oxygen. The shelf life of sample at room temperature should be held as short as possible.

2.7 Concentration and drying procedures

Obtained sample solvents were fully evaporated and concentrated by applying the drying operations described below. Techniques were selected to prevent the thermal decomposition of bioactive component in aqueous and organic solvents.

- For small sample-volume (milliliter) the SpeedVac-concentrator (Savant SC 110 SpeedVac) was used. The instrument consists of evacuated centrifugal tubes. It is designated to accommodate glass, plastic and micro tubes. From the opened centrifugal tubes the solvent evaporated by connecting the oil high performance vacuum pump (Savant instruments Inc, model; VLP 120) of about 0.01 hPa. Centrifugal force keeps samples from pumping, preventing sample loss and resulting in superior analyte recovery.
- For large amount of aqueous samples the liophilisation "freeze-drying" (Lyovac GT 2, Leybold-Hereaeus) was used as a concentration technique. In this method the drying samples were obtained by the deep freeze of the aqueous samples in an oil vacuum pump (RD 4, VacUUrand) at approximately 0.01 hPa. In order to prevent produced sample particles from vaporisation, the sample vessels were closed with punched parafilm
- For concentration of samples in light volatile solvent, distillation under low pressure in a rotary evaporator was used.

It was important to use these methods because they evaporated the solvent and formed small-volume sample with a great efficiency.

2.8 Separation-operations

2.8.1 Centrifugation

For separation of light sediment solid component from sample solution, a Hettich table centrifuge (EBA III) was applied at room temperature in most cases. Furthermore, the sedimentation operation of centrifugation was performed to obtain the clear sample solution by preparative cooling centrifuge (Minifuge GC, Heraeus christ).

The combined extracts were centrifuged in vacuum cool centrifuge (Minifuge GC, Heraeus christ) (4000 rpm, 4°C, 10 min) and the liquid supernatant was carefully pipetted. After this the solid residues were extracted 3 times with 5% diluted acetic acid and then centrifuged. The combined liquid supernatants

were bioassayed and stored at –25°C prior to the drying process. Finally, the residues were dissolved in Millipore water and bioassayed.

2.8.2 Ultrafiltration

In order to separate the macromolecular components from liquid biological matrix and to evaluate the molecular weight of biological active substance, an ultrafiltration method was applied.

Ultrafiltration system makes it easy and practical to separate large and small biological molecules. The 50 mL ultrafiltration cell (Amicon) with filter membranes (Diaflo Ultrafilters, Amicon) was used.

The stirring assembly with a magnetic stirrer was derived at room temperature under the nitrogen pressure of 3,5 Pa by 500 rpm. The first filtration step occurred through the membrane with the size of 10 kilodalton (Ultrafiltration membrane YM 10, 43 mm, Diaflo), which resulted in a light yellow solution. For the next filtration step, a membrane with the size of 1 kilodalton (Ultrafiltration membrane YM 1, 43 mm, Diaflo) was used and a clear solution was obtained. With each step the filtrate was collected in a glass bottle kept in an ice bath. The residue from the filtration was rinsed with water and pipetted off. The filtrate and residual sample solutions were then examined in the bioassay for their contents of biologically active components.

2.9 Chromatographic methods

2.9.1 Initial consideration to the selection of methods

In order to achieve the optimal chromatographic selectivity for an analytical separation problem, the correct choice of adsorbents (stationary phase) and of suitable elution solvents (mobile phase) must be confirmed by experiments.

The selective stationary phase adjusted to appear the adsorption interaction with the polar hydrophilic sample component. On the basis of quality of the diluted sample as elutes polar solvents (such as water or water containing solvent mixture) must be used. Selective liquid chromatographic methods allowed the separation of isolated biological substances in different fractions in

which investigated bioactivity was located via bioassay. In addition to separation, the important hint on the physical quality of examined substance obtained from the retention behaviour. To separate the pheromone containing mixture substance the methods of high-performance liquid chromatography, gel chromatography and thin layer chromatography were used.

2.9.2 Detection

The selection of a detector for the analysis of a particular sample depends on the structure of the analyte and the interferences that may occur from other components of the sample or the eluent. Ideally, in this mode, UV detector and photodiode array detectors were individually used to detect the separated sample at the end of the column. This Spectrophotometric detection can be regarded as the standard method for the separation of biological active component.

System I:

In HPLC as a UV detector UV/Visible 916 spectrometer of GBC Company was used for detection. It was mostly operated at a single wavelength 200 nm whose UV cell should have very small volume (8 µL). Data acquisitions were accomplished by using the software (GBC spectral, version 1,50) via PC (Starchip computer profiline). The spectrums were measured with a scan speed of 500 nm min^{-1} at a gap length of 2 nm. All of the measurements performed absorption within the wavelength region from 200 to 400 nm. Sample and reference were measured in UV permeable Quarz glass-cuvette (500 µL, 10 mm, Hellma). As reference, pure solvent was selected. The eluted fractions in the region of biologically active component obtaining from the HPLC or GLC were determined the characteristic UV absorption spectra. In addition to the optical qualitative evaluation of solution of the chromatogram and chromatographic purity of fractions, the spectroscopic purity of eluted fractions could be stated as additional criteria.

System II:

Photodiode array detector (PDA) (Waters 996) which allows the obtaining of UV/VIS spectra of the corresponding peaks from the same chromatographic run recorded continuously monitoring the full absorption spectrum at two wavelengths of 200 and 254 nm. The data information obtained with a PC (Digital) by the Millenium 2.15 software was received as 3D or contour-line presentation. In this case it can provide much more information about the composition of the sample than with monochromatic detection. Photodiode-Array detector was used as flow-through detector for HPLC due to his high recording speed based on the multichannel advantage. In the time interval of 1.5 seconds, complete UV spectra of the eluted compounds can be recorded, not just LC peaks measured at a single wavelength (Kellner et al., 1998).

2.9.3 High-performance liquid chromatography (HPLC)

In the case of high-performance liquid chromatography (HPLC) or rapid liquid chromatography, the separation of interest sample is carried out at the packed separation column. The particle size of packing material would correspond too minimally. As a consequence, the packing material resists the eluent flow and the mobile phase has to be pumped through the column with pressure. The analytical columns are normally 10 to 25 cm long with an internal diameter of 4.6 mm.

On account of the stationary phase there was applied to the chemical modified reversed-phase (RP-phase) that makes use of a non-polar phase and separates compounds according to their hydrophobicity. As a basic carrier, material silica gel is used and the surface of it consists of free silanol- (Hydroxyl-) groups. In order to receive siloxanes (Si-O-Si grouping) as chemically bonded phases it reacts with alkyl chlorsilanes. Different alkyl and aryl side-chains have been introduced onto the silica surface and in practice C_{18} (*n*-octadecyl) and C_8 (*n*-octyl) are most frequently used. As well as alkyl- and aryl-bonded phases, the other side chains (amino, cyano or amide functional groups) with different polarities and interactions can be used. The advantage of RP-phase exists in the possibilities, polar eluent, in particular water, to employ the targeted separation of polar compound. In view of the bioassay, it is very useful.

In this case, elution system may use isocratic (i.e. constant mobile phase strength) or gradient with other solvents such as methanol or acetonitrile or solvent mixture. The pheromonal substance can be separated by the interaction of unpolar stationary surface and non-polar parts of molecules. The participation of hydrophobic interaction is discussed at the retention behaviour; in the case of ionogenic and organic sample components, the influence of pH value is to vary the retention behaviour of combination and to adjust the optimal adsorbat-adsorbent interaction (ion-suppression) (Krstulovic et al., 1982).
Finally, a rapid and sensitive RP-HPLC method has been developed for the quantitative separation of biological active substance from the compound mixture.

2.9.3.1 HPLC system

All the preparative chromatographic separation of this work was performed at two HPLC systems. One was a low pressure gradient system (HPLC pump 420, HPLC gradient former 425, Kontron) that was connected with an UV detector (UV/Visible 916 spectrometer, GBC Company) (see chapter 2.9.2). Flow rates between the 0.5 $mL \cdot min^{-1}$ and 10 $mL \cdot min^{-1}$ can be used. Sample injection system must allow volumes in the range of 20 to 500 µL to be introduced. For sample introduction using a microlitre syringe, the sample solution is fed in through a needle inlet into the loop of a 6-way valve.
As the other system, the instrument of Waters company was used. This instrumentation for HPLC consists of three parts: (1) controller (Waters 500) with the multi-gradient system (module 616), (2) an auto-sampler (Waters 717) and (3) a photodiode array detector (PDA) (Waters 996). The chromatographic data acquisitions were monitored by the Millenium chromatography manager 2.15 software. This system allowed the simultaneous acceptance of UV spectrum in chromatography by the multi-wavelength detection. In contrast to the first system, sample introduction was done by an automatic injection system.
In the application of HPLC two different types of columns, for analytical and semi-preparative separation, were available. Most of this work HPLC separations were carried out using analytical columns with sample volumes between 20 to 200 µL and flow rates of 0.5 to 1 $mL \cdot min^{-1}$. Secondly preparative columns with sample volume 50 to 500 µL and eluent flow rate of 2 to 6 $mL \cdot min^{-1}$ were

used. In all experiments mobile phase was degassed in an ultrasonic bath before use. All the procedures were carried out at room temperature.
For the separations the following kind of columns according to the different reversed phase materials were employed:

- Lichrospher 100 RP 18 e, 250×10 mm, 10 µm (Merck)
- Lichrosher 100 RP 18, 250×4.6 mm, 5 µm (Merck)
- Lichrosher 100 RP 8, 125×4 mm, 5 µm (Merck)
- C-16 amide, 250×4.6 mm, 5 µm (Supelco)

As eluent polar solvents and their mixtures were commonly used in RP separations as follows.

- Water, isocratic
- Water and methanol 40:60 (v/v), isocratic
- Water and water/methanol 40:60 (v/v), gradient
- Water and water/methanol 40:60 (v/v) with 0.1 % TFA, gradient
- Water and water/ acetonitril 40:60 (v/v), gradient

Water was taken from Millipore water (Milli-Q reagent water system, Millipore). HPLC grade methanol and acetonitril were purchased from J.T Baker.

Elution occurred isocratic with water or water/methanol or in binary gradient with water/methanol to increase the elution power for unpolar compounds. By using the RP chromatography, the polar compounds eluted first and the more polar the mobile phase has been set, the more strongly the unpolar substances were to be retained. Thus RP-HPLC most likely provided optimum retention and high selectivity separation of the biological active component and related biological inactive compounds.
Nevertheless, to make the good reproducibility of the RP chromatography, after every 5 times injections the column used was cleaned up with the same solvent system.
In this mode, as well as developing an efficient separation of related biological compounds, the synthesised substances were also detected under identical conditions. By comparing the retention times of separated bioactive compo-

nent with the proposed standard substances, it could be obtained the pre-information of the interest active sample to be analysed.

2.9.4 Gel filtration chromatography (GFC)

In this analytical technique, analytes are separated on a porous column packing material on the basis of molecular size. The large molecules, which are difficult to enter the stationary phase, are completely excluded from the gel. Thus the observed biological substance can be readily separated and can be quantified from complex sample matrix.

In this framework, a polymer phase (copolymer from ethylenegylcol and methanecrylat) was used (TSK-fractogel HW40-S resin, ToyoHaas; 20-40 µm particle size, 3000 dalton molecular weight ranges). The pressure- stable glass column (Superformance, Merck; 600×16 mm) was packed with the gel mentioned under pressure and connected with the HPLC- and detector system. As described above, the detection affected with the UV-spectrometer at 200 and 254 nm. As eluent, water was used under isocratic condition with a flow rate of 1 $mL \cdot min^{-1}$.

2.9.5 Thin layer chromatography (TLC)

As a high sensitive method thin layer chromatography can also be used to check the chromatographic purity of a substance in an attempt to separate and identify quantitatively one or more of the components present.

Furthermore, chemical functional information related to the separated sample component can be obtained by the post chromatographic proving process and derivatisation reaction at TLC plate.

In TLC the liquid sample was placed at the end of plate, and this end was immersed in the mobile phase, which was then drawn up the plate by capillary action. The tank used for this operation was usually rectangular. After the front of the mobile phase had reached a short distance from the top of the plate, the plate was removed and the eluent was allowed to evaporate. The samples that remain as spots on the dry plate were detected either visually or by indirect visualisation after chemical treatment to locate coloured spots.

As the stationary phase aluminium sheets (cellulose F, 0,1 mm thick, 20×20 cm, Merck) were used for analytical separation. Multiple samples can be applied to a single plate 1-2 cm apart. The samples were applied to the plates as small round spots at 0,2-0,5 µL using glass capillary micropipets.

For the activity of plates, they should be stored in evacuated desiccator to prevent moisture from affecting them.

The mobile phase used in TLC depends on the samples as follows:

Solvent	Spec.	Diluted ratio
n-butanol acetic acid water	p.a. p.a. millipore	20:3:7
acetic acid water	p.a. millipore	1.5:28.5

Table 2.1. Eluted solvents for TLC

If the analytes can not be examined under the ultraviolet light directly, the following reagents in table 2.2 are applied for post-chromatographic derivatisation.

Reagent	Application	Response (spot)
Iodine vapour	Unsaturated organic and N-contained substances	Brown
10 % sulphuric acid in ethanol	All organic compounds	Black or brown

Table 2.2. Application of reagents in TLC.

When developing TLC by using iodine crystals, its colour reaction was the easiest way to visualise the substance. A few crystals of iodine, already vaporised were placed in the tank. The dried plate to be visualised was dipped into the iodine-containing tank for 5 min. Located substances normally turned brown. Sulphuric acid was also used for the visualisation of organic compounds. After spraying or dipping with sulphuric acid solution, proving sub-

stance was heated at approximately 110°C and reacted to form brown or black spot (Touchstone and Dobbins, 1978).
By measuring the retention factor (R_f) the detected substance could be characterised, and by means of the corresponding colour reaction the purity of the substance concerned could be discovered.

2.10 Characterization methods

After the purification of the biologically active substance from a mixture of components by chromatographic methods, as spectroscopic characterization methods UV-spectroscopy, NMR-spectroscopy were frequently used to identify the pure compound. These methods have the advantage that the spectroscopic parameters are accessible without loss of substance. As a last characteristic method mass-spectrometry was also used for identification of unknown compound.

2.10.1 UV-spectroscopy

The important use of UV-VIS absorption spectroscopy is in qualitative analysis of biologically active compounds due to its sensitivity and comparison with others authentic compounds. UV-VIS spectra were obtained by an UV-spectroscopy, GBC (UV/Visible 916 spectrometer) and photodiode-Array detector (PDA).

2.10.2 Nuclear magnetic resonance spectroscopy (NMR)

The main field of application of nuclear magnetic resonance spectroscopy is the determination of the structure and functional group of the chromatographically isolated substance. Therefore, nuclear magnetic resonance spectra are of great importance in structural elucidation of unknown active compounds Thus NMR method was selected to obtain the spectroscopic sample component. In this case, as nuclides protons (^1H) and carbon-13 (^{13}C) are mainly used. In ^1H and ^{13}C NMR, tetramethylsilane (TMS) was employed as an exter-

nal reference compound. If more information is needed, this may come from correlated spectroscopy known as two-dimensional (2D) NMR (such as $^{1}H,^{1}H$-COSY, $^{1}H,^{13}C$-COSY) in which ^{1}H chemical shifts along both frequency axes are correlated with each other. To measure NMR, a relatively large amount (0.5-5.0 mg) of the sample must be prepared. On account of the solubility of measured samples D_2O (deuterium oxide) (99.9% D, Sigma) and deuterated DMSO (dimethylsulfoxide-d_6) (99.8% D, Deutero) were used as solvents.
The sample to be measured was dried from the solvent. Then the sample was dissolved in 500 µL D_2O or DMSO. For all the NMR spectrums, an AMX_R 500 spectrometer (500.13 MHz for ^{1}H, 125.77 MHz for ^{13}C) of the Bruker Company was used.

2.10.3 Mass spectrometry (MS)

In order to identify the characteristic unknown compounds, which are polar, non-volatile compounds, must be derivatised prior to analysis by conventional mass spectrometry. The chemical derivatisation process is incomplete, reduces the amount of useful structural information pertaining to the compound of interest and is difficult to approach the characterisation of unknown sample. For these reasons, it is advantageous to choose the mass spectral technique that does not require derivatisation before analysis (Hettich and Jacobson, 1995).

Selected technique is also performed as sensitive and high specific method to compare the identity of isolated biological active substance with synthesised substance. In fact, for determining the molecular masses of substances, a new ionisation technique, which appeared to be well suited for the direct structural characterisation of non-volatile compounds named MALDI-TOF (Matrix-assisted laser desorption/ ionisation – time of flight) mass spectrometry with a Bruker–Reflex III have been employed in reflectron mode using delayed extraction (Edmondson and Russell, 1996). Sample ions were generated using a 337 nm wavelength nitrogen laser with pulse duration on the order of 3 ns. For this purpose no matrix was used to be analysed. A detailed description of instrumentation of MALDI-TOF technique was given by Hillenkamp and Michael (1991) and Kellner et al. (1998) and Bruker Refex user´s guide. If the sample

was completely separated by using fractionated chromatography, these pure bioactive fractions can be collected for LDI-TOF (Laser desorption/ ionisation – time of flight) analysis. For acquiring averaged mass spectra 20 µL of concentrated sample was spotted on the sample plate for analysis. An aliquot of sample already to be dried was then assisted by rotatory evaporator at about 200 hPa. All samples were detected in positive- and negative-ions mode. The instrument was set to the selected parameters and the software started the scan. The laser was fired and a number of mass spectra was averaged.

2.11 Preparation of standard compounds

In order to compare the chromatographic and spectroscopic data of the natural substance with those of proposed compounds, these proposed standard compounds were required. It was not possible to obtain these compounds commercially . Therefore, the main of this chapter is on synthetic procedures of proposed compounds.

2.11.1 Preparation of standard 1 compound

In order to verify the proposed structure, ichthyopterin identified as 6-(1,2-dihydroxypropyl)-isoxanthopterin named also 7-hydroxybiopterin was synthesized according to a known procedure (Tschesche and Glaser, 1958). According to their procedure it was firstly prepared by synthesis from 6-acetoxyisoxanthopterin.

5 g of 6-hydroxy-2,4,5-triamino pyrimidine sulfate (0.019 mol) dissolved in 113 mL hot water was treated dropwise with 2.8 g of ethyl-acetooxalate (0.018 mol) and 4 mL morphine in 8 mL methanol. The reaction mixture was refluxed with stirring for 10 min. After that the orange color suspension was immediately achieved and then cooled at room temperature. After cooling the crystals were collected by filtration and washed with water, methanol and ether solvents. The product 1 achieved 2.8 g (62.7%).

For second step, the suspension product from step 1 was used as a reactant. 1.175 g of product 1 and 0.555 g of seleniumdioxide in 200 mL acetic acid was heated for 5 hr at 70°C under the reflux. Then the temperature of reflux was

raised to 90°C for 2 hours. The warm reaction mixture was immediately filtrated to remove the selenium particles and then the filtrate was concentrated by using the rotary evaporator. During this evaporating, the crude product 2 with yellow color was obtained.

For the third step, this suspension yielded 2 dissolved in 100 mL 0.1 N Na_2CO_3 and was treated with 150 mg $NaBH_4$ to give the reduction reaction. It was stirred under reflux for 2 hours and acidified by acetic acid. After acidification the reaction mixture was boiled and then immediately warm-filtrated. The resulting filtrate was cooled; this crude sample was the final authentic sample product. It was concentrated, using a rotary evaporator, to 20 mL greenish yellow crude. To obtain the analytically pure sample it was examined by using the RP-HPLC and UV measurements. From the final HPLC separation, 4.6 mg of pure substance was obtained.

2.11.2 Preparation of standard 2 compound

In this case, two steps of synthesis were carried out according to a procedure described by Sprison (Sprison and Chargaff, 1946) and Tschesche (Tschesche and Glaser, 1958).

5.1 g of 2-oxo-butyric acid (0.05 mol) dissolved in 20 mL toluol was placed in a 3-neck flask equipped with 3 gas flasks. 8 g dry bromine was added drop-wise with stirring under the reflux. The heat of reaction was controlled at 50°C. when the bubbles were shown in the gas flask, the significant bromine reaction was observed. Absence of bubbles in the gas flask was a sign that the bromine reaction was complete and the stirring was then continued until there was no change of color with the litmus paper. The crude product (3-bromo-2-oxo-butyric acid) was used directly for the second step.

3.6 g of 6-hydroxy-2,4,5-triamino pyrimidine sulphate (0.014 mol) in 200 mL boiling water was treated drop-wise with 2.5 g of 3-bromo-2-oxo- butyric acid. The reaction mixture was stirred for 3-4 hours at 70°C. After this, the resulting yellow crystals were filtrated. These suspensions were added with 112 mL 0.1 M NaOH solvent and the active carbons. The reaction mixture was heated to boil for 30-40 minutes and the active carbons were removed by filtration. Although this warm filtrate was poured into the 160 mL 2N HCl, the yellow crys-

tals were not obtained. However, after changing the pH value to 1 the yellow crystals appeared. These crystals were collected by filtration and washed with water and methanol. Filtration produced 3.122 g (100%) of standard 2 as a yellow powder. To obtain the pure compound, RP-HPLC and UV spectroscopy were used to purify the powder.

3 Results

3.1 Isolation of pheromones from *Danio malabaricus*

3.1.1 Sample preparation and storage

Method 4 (see chapter 2.5) has been presented as a suitable method for the preparation of pheromone containing materials. By using this method, the biologically active components were obtained completely from the skin extract.

It has been mentioned that enzyme systems can be freed by method 4. Theoretically it is thought that the hypothesis by releasing the enzyme system would result in a derivatisation in the signal substance chemically, resulting in biological decomposition.
However during the experiment using method 4, it was found that the sample did not change its bioactivities when placed at room temperature for several hours. Therefore, this hypothesis in chapter 2.5 is not valid.
The dissolved water residues of homogenate and filtration gave no response to bioactivity in bioassay. However, the supernatant collected by this method (see chapter 2.5) indicated the content of the active samples. It was found that the extracted compounds with significant biological activities were still bioactive after storing them for 8 months at -25°C.
Dry operations mentioned in chapter 2.7 also allowed the maximisation of the effects of biological activities in all experiments.

3.1.2 Optimising the separation operations

3.1.2.1 Centrifugation

As described in chapter 2.8.1, all liquid supernatants were obtained by using the centrifugation. It showed the active reaction and the residues from centrifugation could be found no behavioural response to biological activity.

3.1.2.2 Ultrafiltration

In this work, ultrafiltration was a suitable process as a membrane separation for the last separation of extract mixtures prior to the HPLC analysis (see chapter 2.8.2). By using the 1 kD and 10 kD membranes, cutoff range of the molecular weight of compound mixtures were 1000 or 10000 $g \cdot mol^{-1}$. This proves that the bioactivity of collected samples did not really change. As described in chapter. 2.8.2, the supernatant was filtrated in ultrafiltration cell by 1 kD membrane and the yellow–greenish filter residue was washed with 5 mL Millipore water. Then this dissolved water residue was filtered again by 1 kD membrane. This washing process was done at least twice. The small amount of Millipore water containing these residues of each ultrafitration was used for bioassay. It could be shown that these residues gave no biologically active reaction.

3.1.2.3 Summary of a partial result

At present work the selected ultrafiltration process was the most useful method for further separation by limiting the molecular weight to 1000 $g \cdot mol^{-1}$ containing the pheromone extracts. After the homogenate of skin extract of 50 fish (see chapter 2.5), an ultrafiltration was used and then combined solutions from 1 kD membrane filter were concentrated by using the rotary evaporator. Moreover, as a further separation step, HPLC was used to isolate the biologically active initial substance from the complex matrix.

3.1.3 High-performance liquid chromatography (HPLC)

If the sample of interest (supernatant) was not sufficient to separate by using the extraction procedures, the subsequent high-performance liquid chromatography was best suited to remove the biologically inactive compounds from a complex mixture. This method is advantageous in devising a sample and separating it into fractions at the different time interval according to the polarity and solubility of the solute molecules. As mentioned in chapter 2.9.3, nonpolar stationary phase and polar mobile phase of solvents were used to achieve the desired separation. By using a more nonpolar stationary surface than the eluent, typical reverse-phase was exhibited. The adjustment of the retention behaviour was achieved by varying the composition of the mobile phase. For the HPLC, the sample obtained from concentrated “acetic acid- water extract” of an ultrafiltration was inserted.

3.1.3.1 Analysis of the retention time of pheromone

A factor of considerable interest in separating a biologically active component from complex compounds was the retention time required to perform RP-HPLC. The main objective was to achieve the best separation in minimum time. It could be obtained by using the selected system (for example, column: RP18-LiChrospher, 250×10 mm; flow rate 6 $mL \cdot min^{-1}$) and by variations of the solvents.

3.1.3.2 HPLC separation step (1)

In the first separation step, ultrafiltered concentrated complex compounds were introduced at the reverse-phase column material with a methanol-water gradient system. The entire aqueous methanol elutes were fractioned, dried in a rotary evaporator (35 hPa, 30°C bath temperature) and stored at -25°C until used for testing or analysis.

The chromatograms of the biological matrix solutions were carried out with selected parameters shown in table 3.1. They were then fractionated after detection of the signals. The first HPLC separation of initial substance containing pheromone is shown in figure 3.1.

CHROMATOGRAPHIC PARAMETERS			
Column	LiChrospher 100 RP-18 endcappend, 250×10 mm (10µm)		
Mobile phase	A: Water B: Methanol:Water (60/40) (v/v)		
Gradient	t (time)	%A	%B
	0	100	0
	3	100	0
	13	35	65
	13	100	0
	22	100	0
Injection volume	500 µL		
Flow	6 $mL \cdot min^{-1}$		
Detection	UV, λ = 200 nm (wavelength)		

Table 3.1. Parameters of HPLC system to fractions

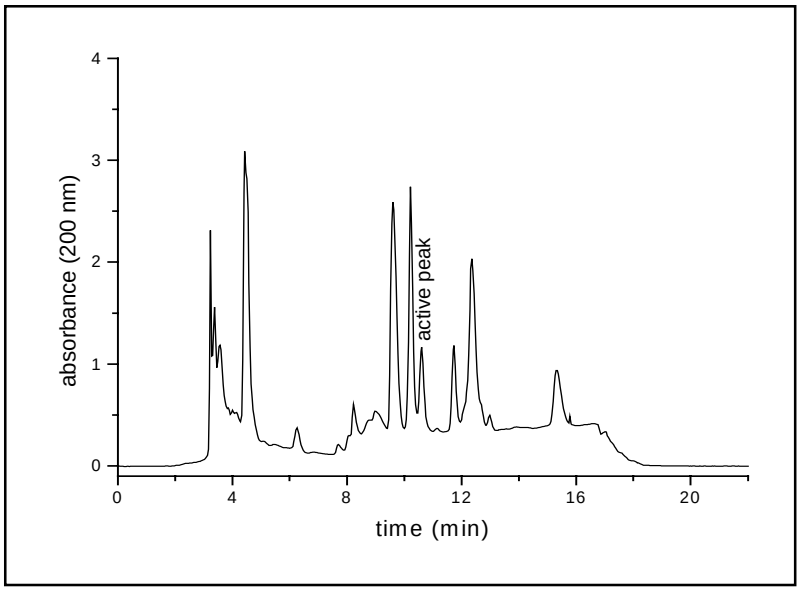


Figure 3.1. First chromatogram of biologically extract sample

The sample fractions began right at the start. As RP-HPLC fractioning, full set of fractions were collected and bioassayed. A total of 8 fractions were obtained during 22 min (W1.1, W1.2, W2.1, W2.2, W3.1, W3.2, W4.1 and W4.2).

3.1.3.2.1Bioassay of fraction W1-W4

To prove the bioactive substance all the different fractions collected from the first HPLC separation step were used for bioassays. As mentioned in chapter 2.4.1, by using these parameters the bioactivity of fractions are confirmed in table 3.2.

Fraction	T_R (min)	Test volume	Test number	Bioassay result
W1.1	0.0-0.5	2 mL	3	$(- -)^3$
W1.2	0.5-8.7	2 mL	3	$(- -)^3$
W2.1	8.7-9.3	2 mL	3	$(- -)^3$
W2.2	9.3-11.6	2 mL	4	$(- -)^4$
W3.1	11.6-11.9	2 mL	5	$(+ +)^4 (+)^1$
W3.2	11.9-13.4	2 mL	5	$(- -)^4 (+ -)^1$
W4.1	13.4-15.9	2 mL	4	$(- -)^4$
W4.2	15.9-22.0	2 mL	3	$(- -)^3$

(- -): negative reaction; (+ -): questionable reaction; (+): positive reaction (no. 2); (+ +): positive reaction (no. 1); T_R: retention time of 1.chromatogram; superscript of bracket: numbers of tests

Table 3.2. Results of bioassays

These tests gave the biologically active fraction (W 3.1) a retention time between 11 and 12 minutes. The chromatogram in this region exhibited an intensive signal (see figure 3.1). According to the first chromatogram, the pheromone-containing materials were separated with methanol eluent. On the basis of these conditions eluted pheromone could be a polar component. This short retention time in the first chromatogram showed evidence of the effect of phase equilibrium with methanol. Considering this short pheromone retention

time, it was possible to separate the large parts of biologically inactive compounds from the acetic-water extract.

The UV spectrum of fraction 3.1 showed a characteristic spectrum so that the selective pheromone isolation could be carried out by these chromatographic parameters.

With water eluted samples, fractions W1.1, W1.2, W4.1 and W4.1 were biologically inactive. With methanol eluted compounds, fractions W2.1 and W2.2 did not develop any significant behavioural changes either. As a result of these bioassays, it was indicated that the bioactive component was fully obtained only in fraction 3.1. All of fraction 3.1 from the first chromatogram was collected with the same system and then dried in a rotary evaporator until its solvents were mostly removed. The highly concentrated sample solution collected from fraction 3.1 was used for next RP-HPLC separation.

3.1.3.3 HPLC separation step (II)

To optimise the rechromatography in the second separation step, the concentrated fraction 3.1 was eluted through the use of two different chromatographic systems of similar parameters (see table 3.3). According to the criteria of the signal retention time, chromatographic solutions were fractionated.

Chromatographic parameters			
Column	LiChrospher 100 RP-18 endcapped, 250×10 mm (10µm)		
Mobile phase	A: Water B: Methanol:Water (60/40) (v/v)		
Gradient	t (time)	%A	%B
	0	100	0
	3	100	0
	13	35	65
	13	100	0
	22	100	0
Injection volume	200 µL		
Flow	3 mL·min^{-1}		
Detection	UV, λ = 200 nm (wavelength)		

Table 3.3. Parameters of second chromatography

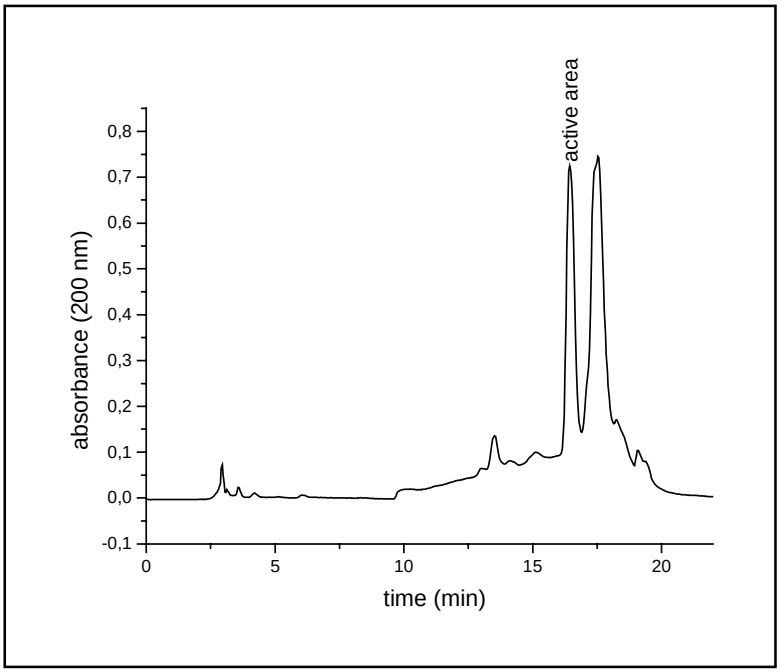


Figure 3.2. Chromatogram of fraction 3.1

By using the mentioned qualitative parameters (see chapter 2.4.1), these 3 fractions were used to examine the bioactivity of the fractions.

Fraction	T_R(min)	UV-maximum
3.1.0	0.0-16.16	no
3.1.1	16.16-16.9	248.6 and 343.2 nm
3.1.2	16.9-19.0	252.2 nm

Table 3.4. UV maximum of fraction 3.1.0, 3.1.1. and 3.1.2

3.1.3.3.1Bioassay of fraction W 3.1.0, 3.1.1 and 3.1.2

The bioassays of the 3 fractions were derived experimentally. These were demonstrated by the presence of a fright reaction to fraction 3.1.1(see table 3.5).

Fraction	T_R (min)	Test volume	Test number	Bioassay result
3.1.0	0.0-16.1	1 mL	4	$(- -)^4$
3.1.1	16.1-16.9	1 mL	4	$(-)^1(+ +)^3$
3.1.2	16.9-19.0	1 mL	4	$(- -)^4$
(-): negative reaction (no. 4); (- -): negative reaction (no. 5); (+ +): positive reaction; T_R: retention time of 2. Chromatogram; superscrift of bracket: numbers of tests				

Table 3.5. Results of bioassays

The data of table 3.5 evidently reveals that only fraction 3.1.1 gave a positive alarm reaction. The chromatographic resolution in this region was qualified for the isolation of pure pheromone fractions. UV spectra of the biologically active fraction showed characteristic two absorption bands with a sharp peak at 248.6 nm and with broad band at 343.2 nm respectively (see figure 3.3).

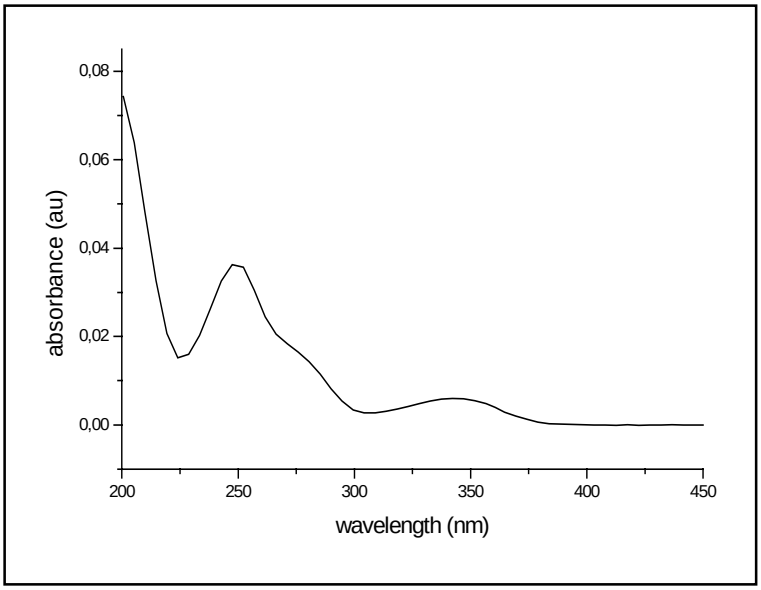


Figure 3.3. UV spectra of biologically active fraction 3.1.1

3.1.3.4 HPLC separation step (III)

The re-chromatography of bio-active fraction 3.1.1 was carried out again under the same parameters (see table 3.1). A highly intensive peak at the same retention time as the second chromatographic step was obtained (see figure 3.4). Then these fractions were collected and the characteristic UV spectra were measured (see table 3.6).

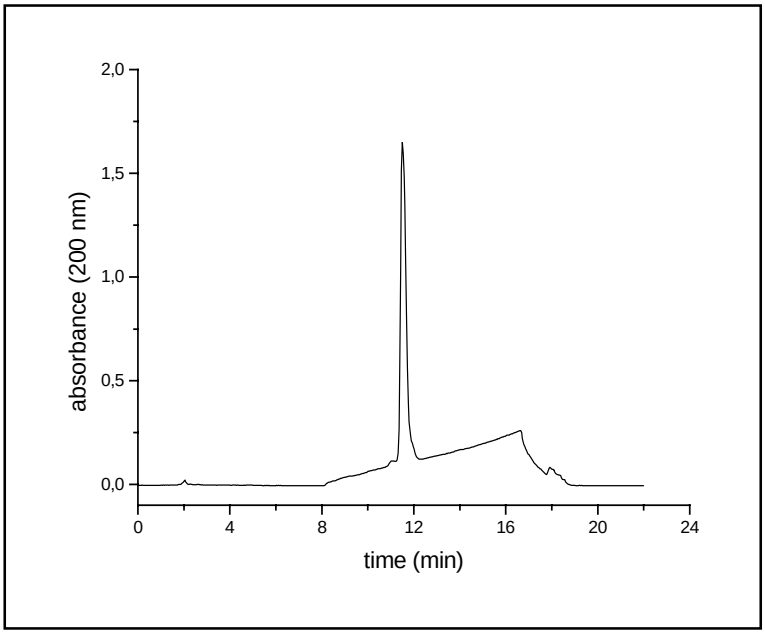


Figure 3.4. Third chromatography of fraction 3.1.1

As in fraction 3.1.1, eluted fraction 3.1.1.1 also showed the same UV characteristics (λ_{max} – 248.6 nm and 343.2 nm).

Fraction	T_R (min)	UV-maximum
3.1.1.0	0.0-11.34	no
3.1.1.1	11.34-12.1	248.6 and 343.2 nm
3.1.1.2	12.1-22	no

Table 3.6. UV maximum of eluted fractions in third chromatography

According to the same UV characteristics and similar retention time (compare with 1.chromatogram), it should be noted that both of them were the same bioactive substances. Both fractions collected were tested on a school of the giant danio.

3.1.3.4.1Bioassay of fraction 3.1.1.1 and 3.1.1.2

Fraction 3.1.1.1 elicited the fright reaction and in contrast fraction 3.1.1.2 was ineffective (see table 3.7). This was confirmed in further analysis that only fraction 3.1.1.1 exactly obtained the biologically active substances.

Fraction	T_R(min)	Test volume	Test number	Biotest result
3.1.1.0	0.0-11.34	3 mL	4	$(- -)^4$
3.1.1.1	11.34-12.1	3 mL	5	$(+ +)^4$ $(+)^1$
3.1.1.2	12.1-22	3 mL	5	$(- -)^5$
(+): positive reaction (no. 2); (+ +): positive reaction (no. 1); (- -): negative reaction (no 5); T_R: retention time of 3. Chromatogram; superscript of bracket: numbers of tests				

Table 3.7. Results of bioassays of fraction 3.1.1.1 and 3.1.1.2

However, it was not possible to separate the eluted fraction 3.1.1.1 further and to isolate the chromatographic pure pheromones in this way.

Nevertheless, excellent separations were achieved by gel filtration chromatography (see chapter 2.9.4).

3.1.3.4.2Separation of fraction 3.1.1.1 with GFC

For a GFC (Gel filtration chromatography) analysis, all of the collected fraction 3.1.1.1 were concentrated into a volume (~ 8 mL) by the rotatry evaporator. The gel chromatographic separation of pheromone-containing concentrated sample was used according to the following parameters (see table 3.8).

CHROMATOGRAPHIC PARAMETERS	
Column	Toyopearl HW40-S, 600×16 mm
Mobile phase	A: Water
Isocratic	0-250 min
Injection volume	500 µL
Flow rate	1mL·min^{-1}
Detection	λ = 200 nm (wavelength)
Detector	UV-Visible

Table 3.8. Gel chromatographic parameters

Gel chromatogram obtained is shown in figure 3.5. There were two fractions (G 1 and G 2). Figure 3.5 represents a typical gel filtration chromatogram of the separation of a fraction 3.1.1.1. Retention time of fraction G1 was 109-134 minutes and of G2 was 134-173 minutes.

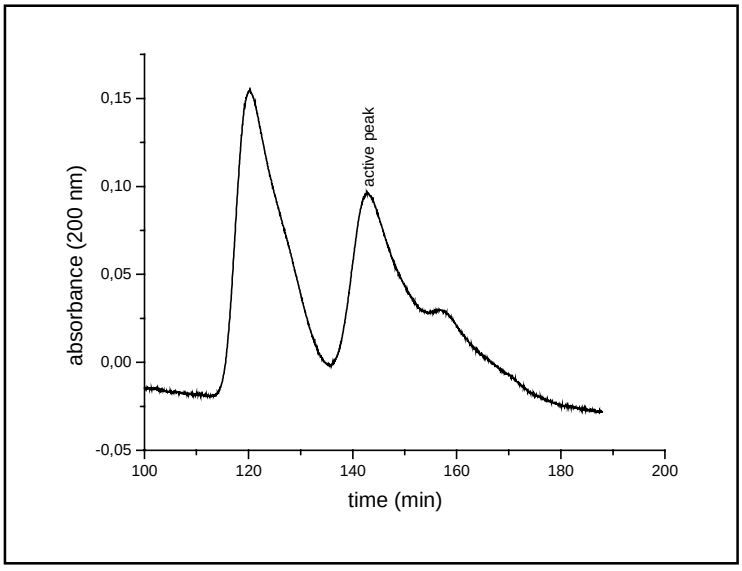


Figure 3.5. GFC chromatogram of fraction 3.1.1.1

The final eluted fractions G1 and G2 were UV spectroscopic pure fractions. Using the GFC method, attractive fraction 3.1.1.1, which could not be separated from each other by RP-HPLC, was successfully isolated into two different compounds as described in figure 3.5. These two major fractions were collected and bioassayed. Their corresponding UV absorption was maximum and bioassay results are given in table 3.9.

Fraction	T_R (min)	Test volume	Test number	Test result	UV maximum
G1	109-134	1.5 mL	5	$(--)^3(+-)^2$	248.6 nm
G2	134-173	1.5 mL	5	$(++)^3(+)^2$	214.6, 290 and 342.2 nm
(+ -): questionable reaction; (– –): negative reaction (no. 5); (+): positive reaction (no. 2); (+ +): positive reaction (no. 1); T_R: retention time of GFC					

Table 3.9. Bioassay results and UV maximum of eluted fraction G1 and G2

Due to this chromatographic elucidation it was possible to determine whether the fright response with actually pheromone component could be or not and to isolate the spectroscopic pure pheromone fraction (G2). This resulted in one fraction (G2) being significantly bioactive, while the other fraction was not active (see Table 3.9). According to GFC separation, molecular size of component corresponding fraction G2 is smaller than that of fraction G1.

UV absorption spectrum of fraction G1 and G2 obtained are described in figure 3.6 in which UV spectra of fraction 3.1.1.1 from third chromatographic step was also compared with those of G1 and G2. Figure 3.6 clearly illustrates that the overlapping of UV spectrum of both fractions (G1 and G2) was sufficiently deviated from the UV spectrum of fraction 3.1.1.1.

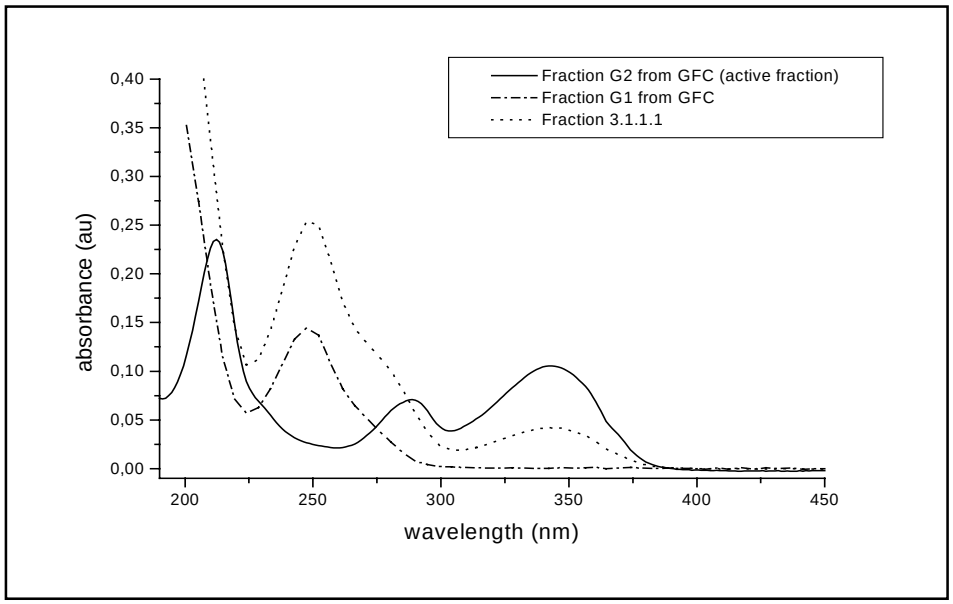


Figure 3.6. UV spectra of fraction 3.1.1.1, fraction G1 and G2 from GFC

3.1.3.5 Summary of pheromone isolation

By using centrifugation and ultrafiltration, within a short time it was possible to achieve a large amount of pheromone-containing sample. In spite of the relatively large column dimension (semi-preparative column), the reversed-phase chromatographic method reduced the analysis time for pheromone isolation. Inactive biologically compounds from complex matrix were removed subsequently by gradient elution and pheromone-containing fraction (UV-248.6 and 343.2 nm) was detected only as a single peak at 3. HPLC.

However, pheromone isolated by the application of these three chromatographic steps required further separation, which could not be attained by RP-HPLC. In this case, with a careful choice of GFC and using Toyopearl HW40-S gel it was possible to separate the two different peaks from a single peak of 3. HPLC separation step. According to the bioassay result, fraction G2 readily gave a fright response and should only be pheromone compound. Their molecular weight would be smaller than 1000 $g \cdot mol^{-1}$ and also smaller than fraction G1.

Complete isolation of concentrated starting materials of 30 mL (50 fish) required about 200 chromatographic separations.

This active sample probably still contained impurities having similar retention times. In such a case it would be necessary to check the purities carrying out other chromatographic column, system and techniques (TLC).

3.1.3.6 Proof of purity

To prove the purity of pheromone-containing substance it was firstly re-chromatographed by using the GLC method under the other HPLC system, which was previously mentioned in chapter 2.9.3.1. By use of a photodiode array detector (PDA), the simultaneous detection of UV absorbance at multiple wavelength was allowed. And it provided to detect the full UV absorption spectrum corresponding to chromatographic peak. This program is given in table 3.9.

CHROMATOGRAPHIC PARAMETERS	
Column	Toyopearl HW40-S, 600×16 mm
Mobile phase	A: Water
Isocratic	0-250 min
Injection volume	200 µL
Flow rate	$1mL \cdot min^{-1}$
Detection	λ = 200 nm, 254 nm (wavelength)
Detector	PDA (photodiode array detector)

Table 3.10. Re-chromatography with Waters instrument

To determine the purity the collected bioactive substance (200 µL) was automatically injected using the above parameter and the chromatogram was shown with flowing rate for one spectrum every second. Figure 3.7 gives the re-chromatogram of fraction G2; it was described as a small impurity peak (retention time 110-135 min) and a large pure peak (retention time 135-180 min). Comparing the retention times and UV spectra of figure 3.5 and 3.7, it can be seen that they are very similar.

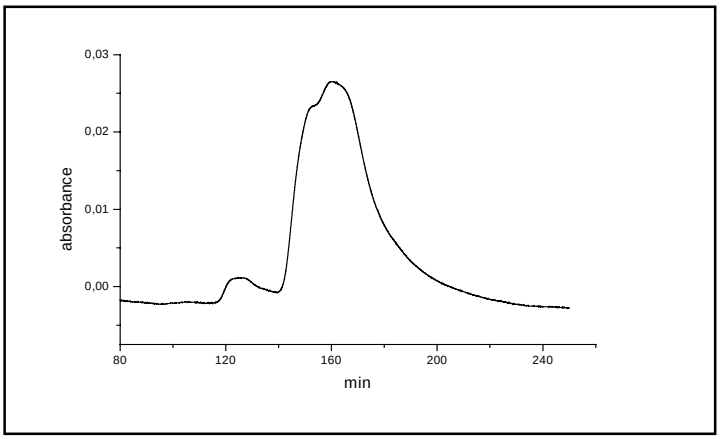


Figure 3.7. Re-chromatogram of fraction G2

The second peak of re-chromatogram (G2.2) was measured for purity. To confirm the purity of the chromatographic peak during the run by using the PDA, their UV absorption spectra were determined every second. After recording the UV spectra, peak purity was confirmed and the region in the retention time of 140 –175 minutes was pure UV spectrum of the second peak was the same as that of eluted G2, which contained the compound of interest. The disadvantage of relatively long chromatographic duration was compensated by the high capacity column.

To check the purity of pheromone-containing sample with another column, the following chromatographic parameters and C-16 amide column were used (table 3.11).

CHROMATOGRAPHIC PARAMETERS	
Column	C-16 Amide, 250×4.6 mm, 5 µm (Supelco)
Mobile phase	A: Water B: Methanol:Water (60:40) (v/v)
Isocratic	95% A, 5%B
Injection volume	30 µL of fraction G 2.2
Flow rate	0.8 $mL \cdot min^{-1}$ in 20 min
Detection	λ = 254 nm, 342 nm (wavelength)
Detector	PDA (photodiode array detector)

Table 3.11. Purity check of pheromone sample

Figure 3.8 shows a typical rechromatogram of biologically active compound. Using PDA, chromatogram was simultaneously detected at 254 nm and 342 nm. Only one signal peak was found in this chromatogram. As shown in figure 3.8, retention time of this active peak was 13.07 minute and UV maximum did not change from that of fraction G2 (214.6, 290 and 342.2 nm). UV spectroscopic investigation showed the characteristic pheromone spectrum. After recording the UV spectra peak purity was confirmed. In addition to the UV spectroscopic purity, this result documented chromatographic purity of pheromone sample.

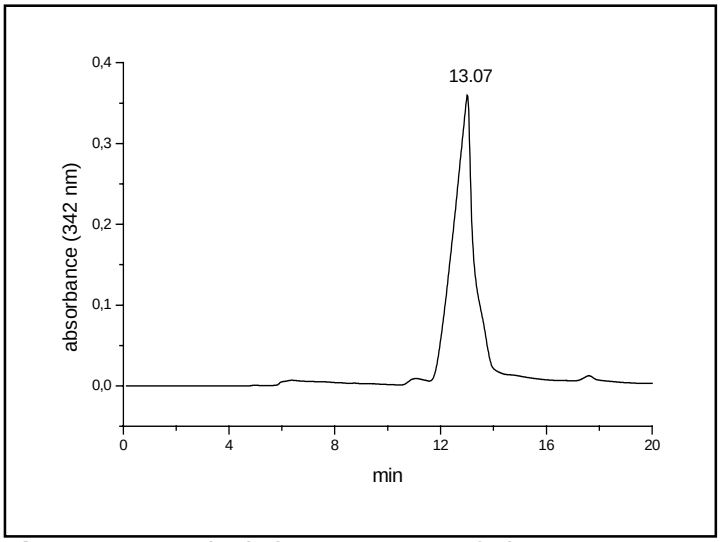


Figure 3.8. Typical chromatogram of pheromone-containing sample

The criterion of chromatographic purity was determined further with the method of TLC.

The concentrated active sample (fraction G 2.2 from GFC) was placed at the end of TLC plate and developed with selected mobile phase and proving reagents. It could be derived post-chromatographically. The results of proving reactions and retention factor of detected substance are mentioned in table 3.12.

Elution solvent	R_f value	Detection
5% acetic acid	0.5	Iodine powder
n-BuOH/acetic acid/water	0.2	Directly detected at 366 nm

Table 3.12. Results of TLC purity check of pheromone sample

As shown in table 3.12, the isolated substance using *n*-butanol/acetic acid/water (20:3:7) solvent mixture as mobile phase was directly detected at 366 nm. However, with 5% diluted acetic acid as the elution solvent, it did not directly show the spot on the plate and thus iodine vapour was used as a proving reagent. In this case, it was not possible to detect the substance using the 10% sulphuric acid as an another reagent.
The isolated substance obtained changed its color to brown after dipping it in the iodine vapour. To analyse the quantity of TLC the eluted biologically active sample was located clearly as only one spot on the TLC plate; substance band proved at R_f= 0.5 (see table 3.12). It was reproduced (repeated) with the same chromatographic systems approximately three times. The R_f values showed no change at all. This bioactive substance, when subjected to TLC, should produce only one spot if it was pure. Therefore, by using the cellulose plate the development of chromatography result proved the purity of the active sample. According to TLC result it was generally suggested that the substance interest may be perhaps one compound. In addition, these results which demonstrated chromatographic and spectroscopic pure substance also evidenced for further analysis of the structure of chromatographically pure compound.

3.2 Structure determination of pheromone

To identify an accurate structure for biologically active component, the chemical pre-information that was considered from pheromone isolation of bioactive mixture compound by using the different spectroscopic methods should be mentioned as follows.

The pheromone substance has a low solubility in water, is poorly soluble in most of organic solvents and has a lack of accurate melting point. It tended therefore to be difficult to crystallize. However, it was soluble in hot water (>70 °C), also in diluted acid solution and basic solution. Therefore, this pheromone substance could be a neutral compound in nature and as yellow-color crystal. The retention behavior of the semi-preparative RP 18-HPLC system was significant under the methanol gradient elution. It was successful to obtain a large retention time in third HPLC and to interact with unpolar and hydrophilic stationary phase.

It acted itself by unsaturated and/or nitrogen atom containing substance. This property was provided by the positive proof reaction of color with iodine vapor on the TLC plate.

3.2.1 UV-spectroscopic analysis of pheromone

UV spectrum is not the only method to prove the identification of the pheromone structure. However, UV spectrum showed that the pheromone consisted of one or more chromophores. Chromophores are structure fragments containing double bonds.

The absorption characteristics of most compounds in the UV region depends on the electronic transitions that can occur in $n\pi^*$ and $\pi\pi^*$ transitions. The $n\pi^*$ transition of single chromophoric groups, such as the carbonyl or nitro group are forbidden and corresponding bands are characterized by low molar absorptivities and are further to a shorter wavelength due to substitution or solvent (hypsochromic shift). The weak $n\pi^*$ band can give proof of functional groups. As long as alkyl groups bind to the heteroatom containing double bonds, for each atom group characteristic absorption regions are found. $\pi\pi^*$ band mostly appears in the spectra of conjugated π electron system and also those of aromatic molecules possessing chromophoric substitution. They are characterized by high molar absorptivity and further to a longer wavelength due to substitution or solvent effect (bathochromic shift) (Silverstein and Webster, 1991).

A relatively large portion of a pheromone molecule may be transparent in the UV so that a spectrum similar to that of a much simpler molecule may be obtained. Thus UV spectrum could serve as a hint for structural identification of the pheromone to present characteristic groups. For example: C-C multiple bonds and/or heteroatom containing double bonds.

The relationship of the absorption spectrum of this pheromone component between 200 to 400 to its structure has been generally predicted aromatic ring structure and in which a band at about 210 to 250 expects the >C=N- characteristic group, last band at longer wavelength is due to a >C=C-C=O- group and a band at about 288 to 290 nm indicates the carbonyl group. UV spectroscopic characters of pheromone were determined by the recording of UV spectrum at different pH values of solvent. From these spectrums it shows that

the structural change of the molecule and UV chromophors depend on the pH values.

All of these measurements were performed in water as solvent and pH values were performed with HCl and NaOH. The UV spectrums of different pH values are described in figure 3.9.

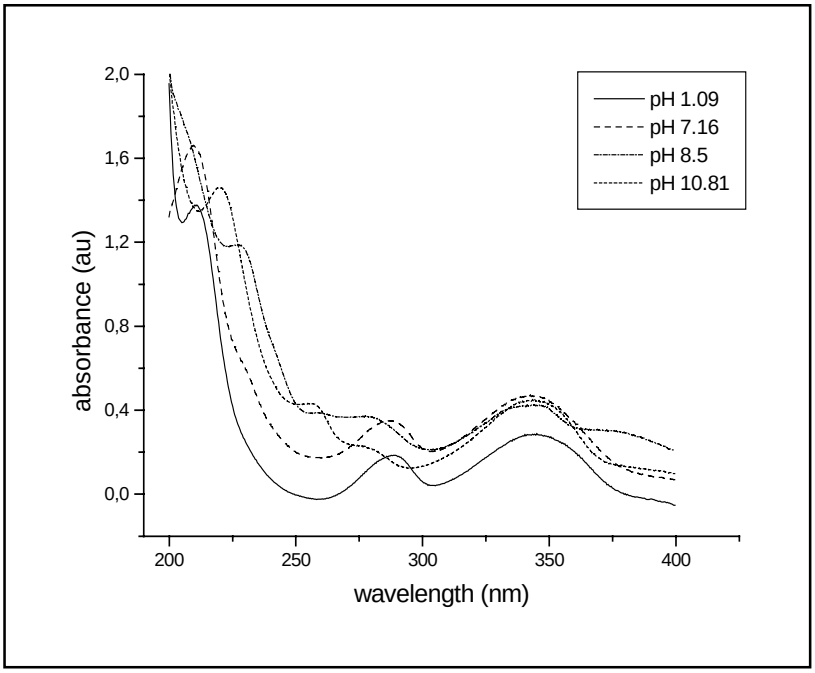


Figure 3.9. UV spectra of active sample at different pH values

As shown in figure 3.9, the pH values of solvents affected the absorption characters. However, the neutral and cation molecules generally resembled in their UV spectra. In the case of mono anion, it had quite different UV absorptions from the neutral molecule. The absorption spectrum of anion (pH = 8.5) usually had 4 maximum bands which are at 228, 280, 340 and 379 (s) nm. The corresponding anion molecule caused the band with a red shift of about 14 nm in shorter wavelength and the band with a hypsochromic shift (blue shift) of about 8 nm in longer wavelength. In the case of dianion (pH = 10.8), there were also 4 maximum bands at 220, 257, 280, 343 nm. This UV spectrum altered slightly from that of monoanion (see figure 3.9). At high pH value, the intensity of absorption at 280 nm was very low, but its absorption frequency could be seen. At high pH value more than 12 their absorption bands become more likely with those of pH 10.8. These characteristic features of unknown component are associated with anion formations at the two-amide groups, which results in a hypsochromic shift compared to the neutral form. This can be explained by reorganization of the π-electron system involving the

two oxo groups. At low pH value, the last two bands (see the spectrum of pH = 1.09) were shifted to shorter wavelength of about 1 nm. This can be seen the protonation of nitrogen atom in the cation form.

The result of this experiment shows that the pheromonal active component could be able to dissociate by using the variation of pH values of solvents. However, this result would not be carried out to deduce the structure of pheromone molecule.

3.2.2 UV-spectroscopic deduced structure hypothesis of pheromones

The characteristic UV spectroscopic data of the pheromone substance obtained from chapter 3.2.1 were compared with the references in the UV data collection (Sadtler research laboratories Inc; BIO-RAD (1973-1994). Thereby the speclocator-index was investigated at the reference values of the UV maximum 212±1 nm, 288±1 nm and 342±1 nm respectively. The variation of absorption wavelength of ±1 nm was considered to get probably a similar spectrum. Comparisons of the UV spectra of the unknown substance with the standard spectra were therefore performed between ±1 nm and this routine checked the absorbance scale at the three maximum points on each standard UV spectra. However, the results obtained from these comparisons did not give any information to identify the biological compound and did not find the identical UV spectrum.

To postulate the prognosis of UV spectroscopic pheromone structure, the UV spectra having three bands with similar absorption characters, similar intensities and similar symmetric bands should be collected and compared. In this condition, to compare the UV spectra, heterocyclic ring system such as purine and pterin derivative substances should be considered, while these relative substances were generally found in fish. Firstly the UV spectra of some purine were studied and their UV spectrum are also dependent on the pH of the solutions (Albert and Brown, 1954). To postulate the structure prognosis of the pheromone UV chromophores, the UV spectra of xanthine, hypoxanthine, guanine and uric acid were investigated. The UV spectra and structure forms

of purines are shown in appendix A1.1 to A1.4 (see page 112-113) and the related UV absorption datas are described in table 3.13.

Substance	λ_{max} (nm)	λ_{max} (nm)	Solvent
guanine	272	213 (s)	methanol
xanthine	276	240	water
uric acid	290	234	water
hypoxanthine	248	198	water

Table 3.13. UV data of some purine compounds

From the UV datas in table 3.13 only two wavelength bands were obtained. In guanine its spectrum found the shoulder with longer wavelength and in xanthine its UV spectrum found a shoulder with shorter wavelength. There are weak n-π bands. These arise from forbidden transitions and are of weak intensity and also show bathochromic shifts in passing from polar and less polar solvents. The others are π-π^* bands and causes in the region of about 220 ~ 230 nm.

In this case of comparison the UV spectra of purine substances and the biologically active compound, the point made concerning the UV spectra of purines could not apply to achieving the structure elucidation of pheromone substance. According to this basis it should be noted that alarm substance might not be a purine derivative compound.

After the comparison of UV spectra of purines, the pterin compounds were used as the second comparison of UV spectra.

The pterins usually have a well-defined UV spectrum with three major bands. The UV spectrum of parent neutral pterin described in appendix A2.1 to A2.5 (see page 114-116), which also had n-π^* bands (250, 270 nm) and π-π^* band (339 nm) was compared with the UV spectrum of neutral pheromone molecule. The result showed that the spectrum of pterin resembled that of the pheromone molecule, although the last two bands of the pheromone were of longer wavelengths. Thus, on the basic of this result, it had a source that the structure of the pheromone compound could be derived.

As expected, introduction of an aliphatic substituent in position 6 or 7 of the pterin nucleus did not alter the physical properties very much, as seen from the biochemically important biopterin and neopterin (see table 3.14). The di-

hydroxy- and trihydroxypropyl side chains cause a small positive inductive effect, shifting the UV spectra of the various molecular species to slightly longer wavelengths in comparison to pterin, making the molecules a little more basic and less acidic, respectively, for the same electronic reason (Pfleiderer, 1985).

The UV spectrum of the 6-hydroxy substituted pterin called xanthopterin (see table 3.14) of neutral form showed the relatively long wavelength band (red shift), and a slight shoulder at 305 nm.

Substance	λ_{max} (nm)	λ_{max} (nm)	λ_{max} (nm)	Solvent
pterin	250	275(s)	339	water
biopterin	234	273	345	water
neopterin	236(s)	274	344	water
leucopterin	240	280	342	water
xanthopterin	275	305(s)	385	water
(s) = shoulder				

Table 3.14. UV data of some pterin compounds

Leucopterin revealed another charactristic UV spectrum in which the additional oxo function made this molecule even less basic. Its cation formation took place only in very strong acid medium. Three maximum wavelengths of UV spectrum of neutral leucopterin (see table 3.14) were 240, 280 and 342 nm. This indicated the small changes of second position from parent pterin.

Another interesting and characteristic feature from pterin derivatives was isoxanthopterin, which was derived from the oxidation of pterin at C-7. It represents a 2-amino-4,7-dioxopteridine form. The UV spectrum of isoxanthopterin (see table 3.15) (appendix A3, page 116) indicated very similar properties to those of pheromone substance as long as the chromophoric system was not altered electronically.

Substance	λ_{max} (nm)	λ_{max} (nm)	λ_{max} (nm)	Solvent
isoxanthopterin	213	288	342	water
pheromone sub.	214	290	342	water

Table 3.15. UV data of isoxanthopterin and pheromone substance

According to Pfleiderer (1985) 6-substituted isoxanthopterin compounds mostly resembled their parent molecules isoxanthopterin in their UV spectra.
To obtain the chemical pre-information about a pheromone by comparing the properties of isoxanthopterin and 6-substituted isoxanthopterins (for example ichthyopterin) it was clearly evident that UV chromophors of bioactive sample were related either to those of isoxanthopterin or 6-substituted compounds.

On the basis of the great overlapping of UV spectroscopic parameter of isoxanthopterin derivatives with the pheromone molecule, it was proposed that the alarm substance is an isoxanthopterin derivatives.

The structure of these isoxanthopterin derivatives include a heterocyclic ring system, an amino group in 2- and hydroxy group in 4- and 7 positions (see figure 3.10). In the derivatives of isoxanthopterin compounds the various substituents are mostly attached to the pterin ring at 6 position (see figure 3.10).

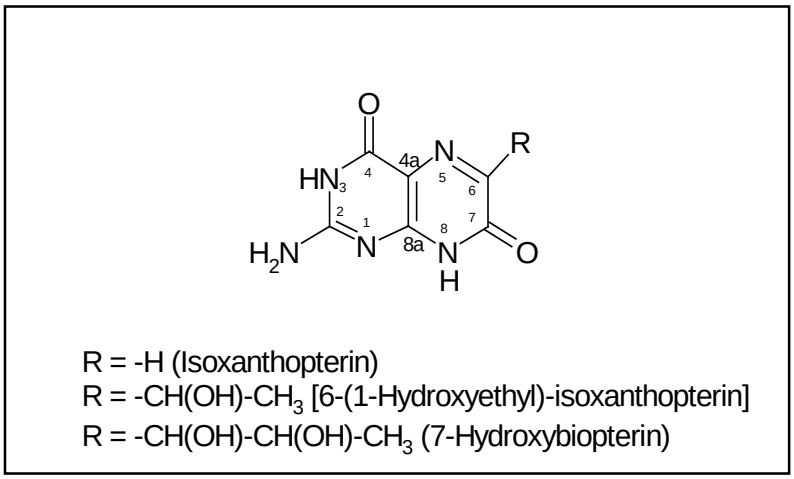


Figure 3.10. Structure of isoxanthopterin and its derivatives

3.2.3 NMR spectroscopic analysis of pheromone

Detailed chemical structure information about the pheromone substance should be obtained from the ^{1}H NMR and ^{13}C NMR measurements.
The analysis sample (~3 mg) was dissolved in 0.6 mL of deuterated DMSO (dimethylsulfoxide-d_6) and was firstly measured for ^{1}H NMR spectrum at 300°K temperature. Chemical shifts were referenced to internal tetramethylsilane (TMS).

The obtained NMR spectrum is shown in figure 3.11 and this NMR spectrum indicated that there might be two compounds. Chemical shift of dissolved water in DMSO-d_6 solvent was found at ~ δ 3.5 ppm, and that of DMSO signal at about 2.5 ppm. A sharp singlet at 3.2 ppm was methanol. In DMSO solution, two doublets at 1.08 and 1.2 ppm were two -CH_3 groups of substituted diol and a proton on the β carbon of diol showed as a multiplet peak about 4 ppm. Another proton on the α carbon a doublet signal was at 4.5 ppm. A small broadened peak at 7 ppm was assigned to proton related nitrogen atom and the two singlet peaks at δ 11.1 ppm and 12.2 ppm were protons at two nitrogen atoms of heterocyclic compound. The region of 11.0 to 12.5 ppm was confirmed for the absorption of NH- protons of amide functions of pterin compounds (Dieffenbacher et al., 1966). However, the hydroxylic protons of two OH groups in substitution were not found in ^{1}H NMR spectrum. It should be noted that these protons might be exchangeable with water or methanol or the rest of other impurities.

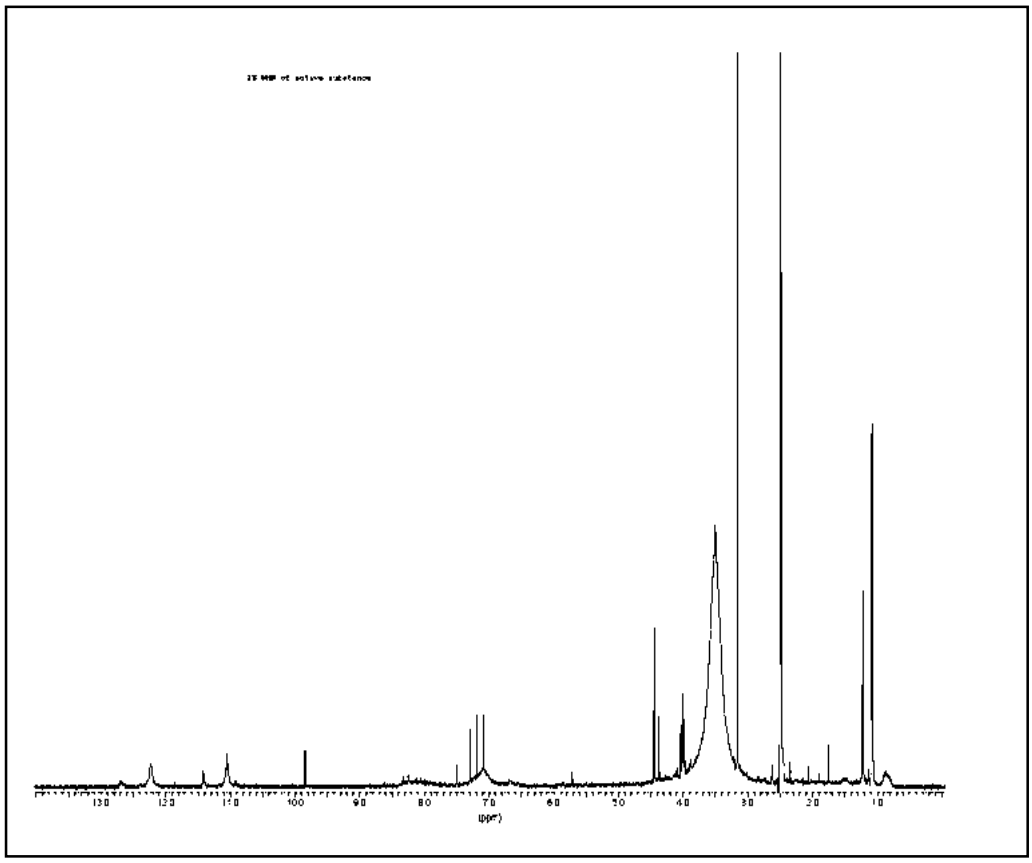
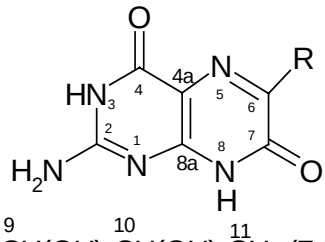


Figure 3.11. ^{1}H NMR of active substance

From the comparison of ^{1}H-NMR spectra of pteridins (Dieffenbacher et al., 1966, 1969) and that of UV spectra of pteridin compounds (see chapter 3.2.2) the structure of basic body of this active compound was generally an isoxanthopterin. The two substituents of the 6 position of this class (see figure 3.12) are interpreted as:



a) R = -$\overset{9}{C}$H(OH)-$\overset{10}{C}$H(OH)-$\overset{11}{C}$H$_3$ (7-Hydroxybiopterin)

b) R = -$\overset{9}{C}$H(OH)-$\overset{10}{C}$H$_3$ [6-(α-Hydroxyethyl)-isoxanthopterin]

Figure 3.12. Proposed structures

These two compounds were found in ^{1}H NMR spectroscopy and when comparing the ratio of integration the main component from ^{1}H NMR should be 7-hydroxybiopterin.

Nevertheless, these ^{1}H NMR parameters were incomplete to interpret definitely the pheromone sample. In this case more information is needed and this may come from additional experiments. ^{13}C NMR spectrum was therefore measured to support the ^{1}H NMR assignments.

The ^{13}C NMR spectrum shown in figure 3.13 contained carbon signals belonging to the one methyl group at δ ~19.6 ppm (C-11 in assignment a), β carbon of hydroxyl group at δ ~ 67.8 ppm (C-10 in assig. a), α carbon of hydroxyl group at δ ~ 73.5 ppm (C-9 in assig. a) respectively. These signals could be attributed to substituted diol group at 6^{th} position on the pterin ring. A peak about 65.7 ppm was probably a substituted carbon atom of α hydroxyl group of second component (C-9 in assig. b). A signal of about 48.5 ppm should be methanol and deutrated DMSO absorbed at 40 ppm. The carbon signal at 110 ppm is expected from the C-4a of isoxanthopterin ring. The other carbon signal groups were found in the range of about 151-159 ppm. According to (Pfleiderer, 1985 and Ewers et al., 1973) the signals at 151, 154.8, 156.8 and 158.6 ppm could be shown as C-8a, C-2, C-7 and C-4 of pteridine ring respectively.

However, it could be possible that the amount of sample was not sufficient to assign all the carbon atoms. In this way, ^{13}C NMR analysis was helpful in characterizing the desired sample. Clearly, the three carbon groups of substituted diol at position 6 confirmed the expected structural assignment (a). (b) could not definitely be found.

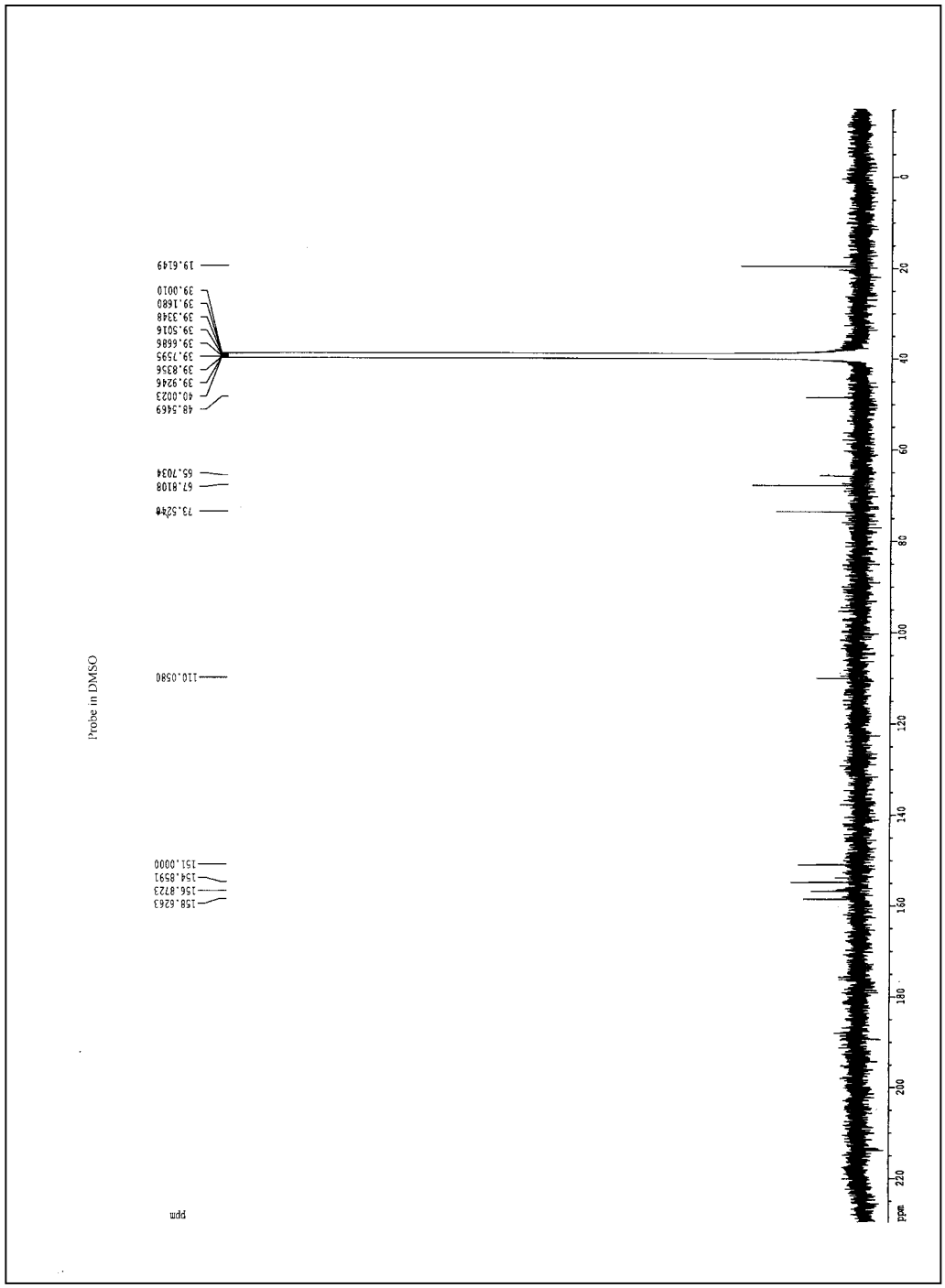


Figure 3.13. ^{13}C NMR of active substance

At this point, using two-dimensional heteronuclear shift correlation measurement (^{13}C-^{1}H-COSY) the structure of the unknown molecule in question could be completely elucidated.

This 2-D spectrum of active sample is presented in figure 3.14. The f_1 (500.13 MHz) and f_2 (125.77 MHz) windows were generally set to cover the entire ^{1}H

and ^{13}C signals, respectively. This means that the abscissa (f_1-axis) corresponds to ^{1}H chemical shifts, the ordinate (f_2-axis) to ^{13}C chemical shifts.

There were two methyl groups at 1.08 and 1.2 ppm. Resonance for two of these methyl groups occurred in the carbon spectrum at 19.6 and 20.8 ppm, thus showing with which protons these carbon atoms were correlated. A multiplet signal corresponding to the substituted hydroxy proton at about 4 ppm was individually correlated with two carbons at 65.7 (very small signal) and 67.8 ppm in which carbons were in hydroxyl groups of assignment (b) and (a) respectively. α-hydroxy proton giving a doublet signal at 4.45 ppm was correlated with the carbon at 73.5 ppm.

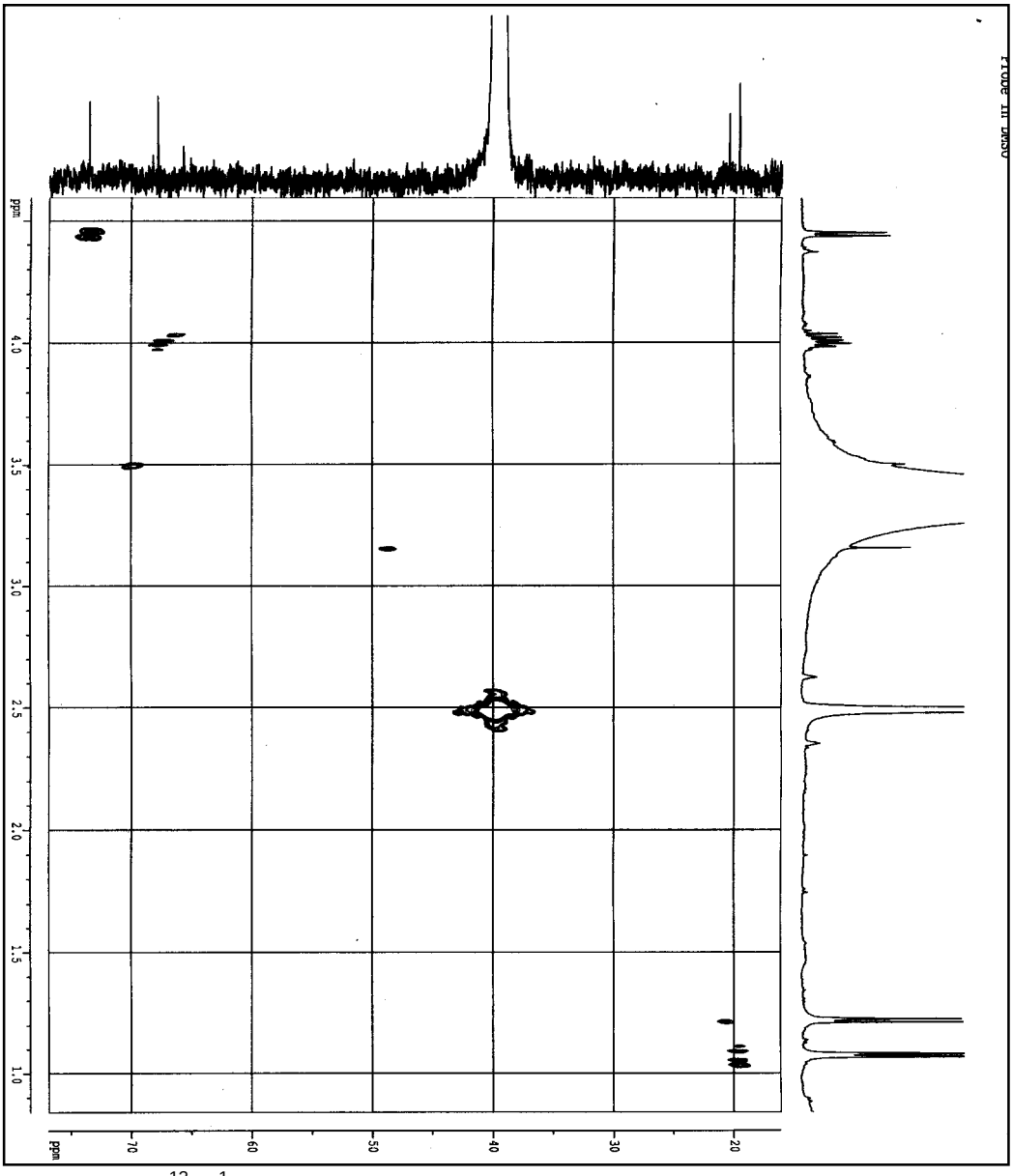


Figure 3.14. ^{13}C-^{1}H-COSY of active sample

These results, compared with the finding of ^{1}H and ^{13}C spectra, agree with those of the previous interpretation, which indicated as two-assignments (a) and (b). In this case the active sample contained a very low amount of component (b). From the NMR analysis it should be concluded that the major compound of bioactive pheromone substance is 7-hydroxy biopterin.

3.2.4 Mass spectrometric analysis of pheromone

The clear proof of the structural identification was carried out with the analytical method of mass spectrometry. Laser desorption ionization mass spectrometry was well-suited for the structural charcterization of non-volatile underivatized compound (Hettich and Jacobson,1995). In this chapter as the last spectrometric determination mass spectrometry was described to appreciate the investigation to the structural elucidation of natural pheromone compound.

A small amount of sample (~ 20 µL) was placed on the sample plate and ionized by laser desorption with a nitrogen laser. The resulting ions (positive and negative ions) were then examined in detail with the LDIMS using accurate mass measurements.

All the mass measurements were used for the same ways; the spectra of active substance are summarized in figure 3.15 and 3.16. The LDIMS produced litte ion fragmentation (McLafferty and Turecek, 1993).

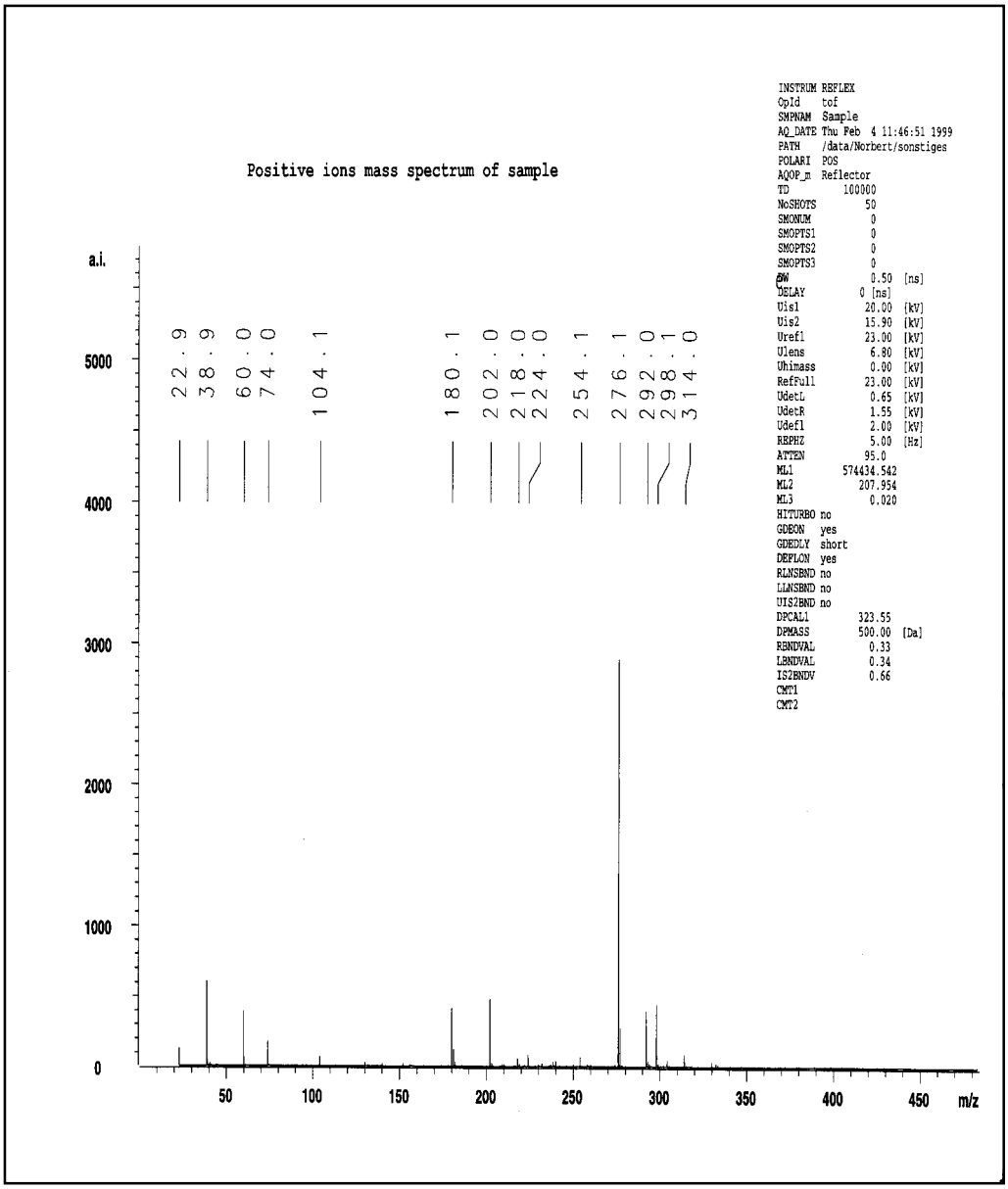


Figure 3.15. Positive ions mass spectrum of active substance

Figure 3.15 illustrates the positive ion spectrum of active substance. These positive ions for unknown active compound consisted of pseudomolecular ions $[M+H]^+$, $[M+Na]^+$, $[M+K]^+$ and $[M+2Na-H]^+$ which were at m/z 254, 276, 292 and 298 respectively. They provided the molecular weight information. According to the positive-ion mass spectrum the mass of compound of interest was inspected. It was possible to verify that the molecular mass of alarm substance is 253. The ion at m/z 180 revealed the loss of the substituent attaching to the 6^{th} position and it was observed identically the molecular mass of isoxanthopterin in the elimination of this unknown substance.

The negative-ion spectrum of active compound is shown in figure 3.16, consisted of $[M-H]^-$ ions and fragment ions. The most possible structure for the $[M-H]^-$(m/z 252) is hydrogen loss from the N^8-position (see figure 3.12). The N^8-hydrogen is known to be the most acidic hydrogen in solution (Hettich et al., 1995).

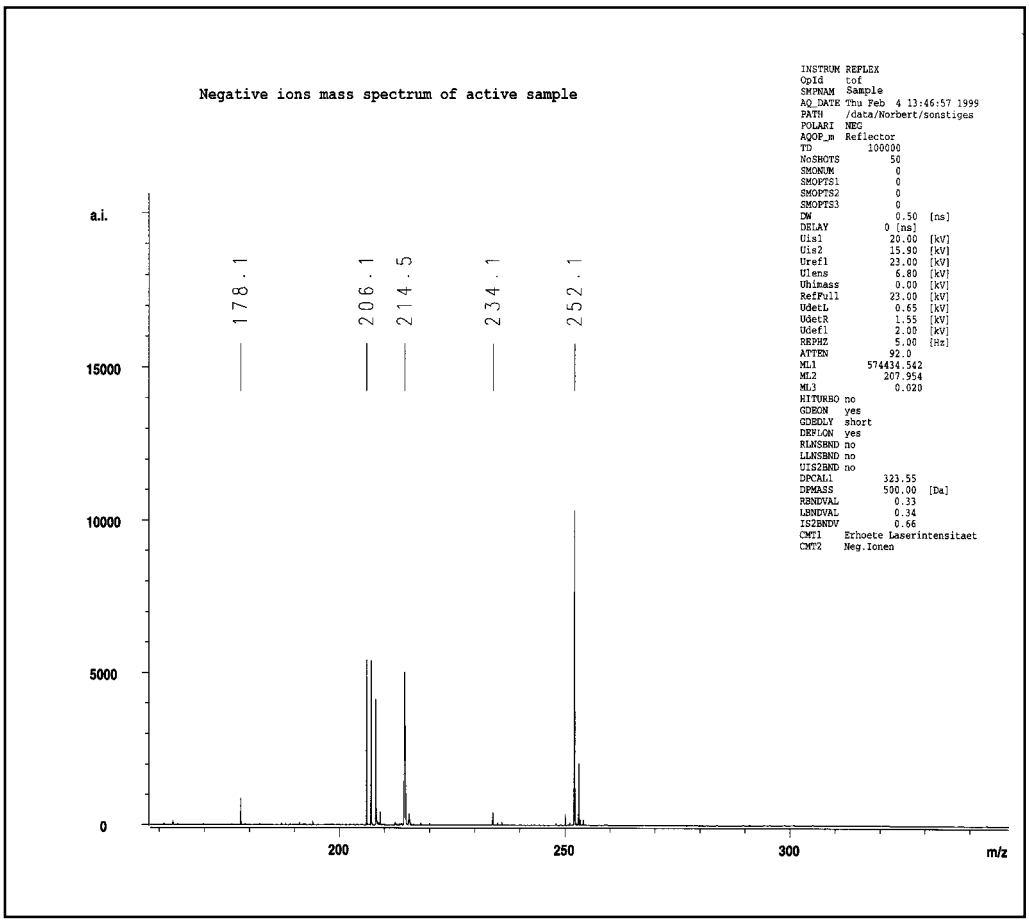


Figure 3.16. Negative ions mass spectrum of active sample

An abundant negative ion at m/z 208 was generated by α-cleavage of the side chain to form the resonance structures shown in figure 3.11. Loss of H_2 from the α-carbon of the m/z 208 ion would generate the m/z 206 ion shown in negative spectrum (see figure 3.17). This m/z 206 ion could dissociate by loss of CO to give an ion at m/z 178 whose structure was also identical with that of isoxanthopterin.

Due to the observation of ion fragments at m/z 180 of positive- and 178 of negative-spectra, isoxanthopterin must be content in the basic structure of the active sample.

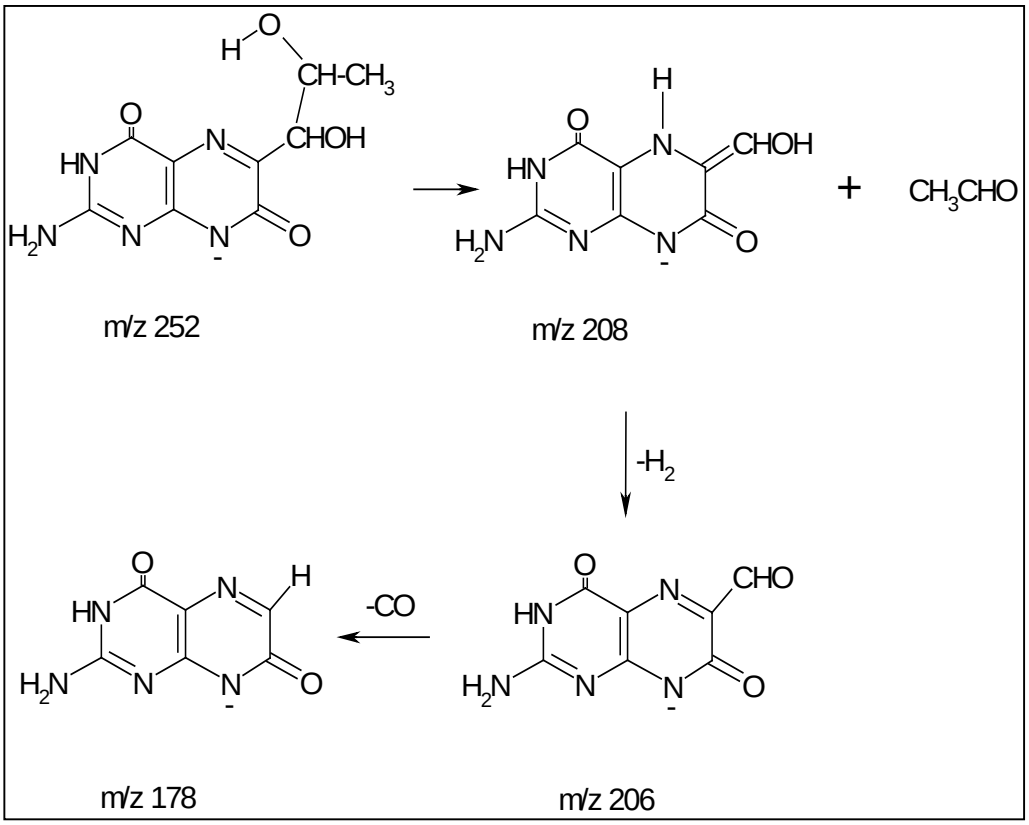


Figure 3.17. Negative-ion fragments of ichthyopterin (7-hydroxybiopterin)

Therefore, the 7-hydroxybiopterin-structure as an assignment (a) of natural substance, which has been derived from UV- and NMR-spectrometric evidence, was fully supported by LDI mass spectrometry.

According to the NMR experiment, assignment (b) should have its molecular weight at m/z 223. However, this structure is not clearly detected by both positive and negative ions.

The active compound was isolated by high-performance liquid chromatography (HPLC) and gel filtration chromatography (GFC), and then this isolated active fraction was identified by UV-Visible spectroscopy, NMR spectroscopy and mass spectrometry for detail analysis. However, structure assignment was possible using these techniques as long as appropriate model compounds were available for comparison. Thus, two model compounds, ichthyopterin (7-hydroxybiopterin) at m/z 253 and 6-(α-hydroxyethyl)-isoxanthopterin at m/z 223, were synthesized as standard compounds (see chapter 3.4 and 3.5). These authentic standards [7-hydroxybiopterin named standard 1 and 6-(α-hydroxyethyl)-isoxanthopterin named standard 2] matched the natural substance in every way.

3.2.5 Bioassays with authentic sample

The experiments were performed in 20 aquaria (40×25×25 cm) containing 20 liters of water. The basic procedures to prepare the all bioassays were mentioned in chapter 2.3.2 and 2.4. The intensity of the fright reaction was evaluated by using the parameters which were described in chapter 2.4.1. Test solutions were prepared from 1 mg 7-hydroxybiopterin and 100 mL Millipore water. The bioactivity of different amount of substance is confirmed by table 3.16.

Test volume	Test number	Bioassay result
20 mL	2	$(+\ +)^2$
15 mL	2	$(+\ +)^2$
10 mL	2	$(+\ +)^2$
8 mL	3	$(+\ +)^3$
5 mL	4	$(+\ +)^3\ (+)^1$
4.5 mL	4	$(-\ -)^3\ (+\ -)^1$
4 mL	3	$(-\ -)^3$
3 mL	3	$(-\ -)^3$
(- -): negative reaction; (+ -): questionable reaction; (+): positive reaction (no. 2); (+ +): positive reaction (no. 1); superscript of bracket: numbers of tests		

Table 3.16. Results of bioassays

As it can be seen in the table 3.16 **,** five mL of solution equivalent to 0.05 mg of 7-hydroxybiopterin elicited the fright reaction in the giant danio. However, bioassays of amount less than five mL did not clearly show the fright reaction.

3.2.6 Verification of UV spectroscopic deduced structure hypothesis

To verify the structural hypothesis of standards 1 and 2, they were compared with the following chemical, physical and biological parameters of the pheromone substance.

The following similarities were discovered:

- retention times at the different HPLC systems.
- characteristic variation of UV spectrum at different pH values of solvents.
- retention factors at TLC plate of different mobile phases.
- solubility in deuterated DMSO solvent for all NMR analysis.
- biological activities.

Since these identical criteria indicated that pheromone substance might consist of two components with standard 1 as the major component, it could be considered without doubt that pheromone structure has a very identical structure to that of standard 1.

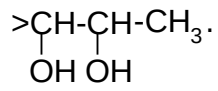
3.2.7 Comparison of the NMR and mass spectra of authentic samples

NMR and mass spectrometry were used to indicate that synthesis of the intended structures had been achieved. The NMR spectral characteristics of standards 1 and 2 are shown in appendix B1 and B2. Mass spectra of these authentic samples are also attached as appendix C1 and C2. It was known that the NMR and mass spectra of these pterins had not been determined. Therefore, they were totally unknown for the spectroscopic analysis.

For all NMR measurements 4.6 mg of standard 1 was dissolved in DMSO-d^6 solvent.^{1}H NMR spectrum of standard 1 clearly showed the proton resonance. Comparison of 1H NMR spectra of standard 1 (see appendix B1.1, page 119) with those of natural substance indicated that the signal groups of about 1.08, 4.0, 4.45, 7, 11.04 and 12.22 ppm were found exactly in both spectra. As described above (see chapter 3.2.3) these chemical shifts were necessary to prove the previous assignment of nearly all signals to specific groups in molecules of interest. However, for more exact information it is important to match the other NMR results (i.e. ^{13}C and 2D-COSY).

^{13}C NMR of standard 1 is shown in appendix B1.2 (see page 120). In this spectrum, the chemical shifts of carbon atoms in standard molecule 1 were fairly found and its ^{13}C NMR spectrum was matched with that of natural substance. This matching showed the carbon signals as being mostly identical

with each other, and clearly supported the previously postulated result that the structure of pheromone was identical to that of standard1. The similar chemical shifts for protons and carbons of the structures were in good agreement with detected data for verification that the 7-hydroxybiopterin is a pheromone compound. Two-dimensional (2D) heteronuclear correlated NMR spectrum of standard 1 (see appendix B1.3, page 121) showed clear correlation of carbon- and proton-atoms. In this 2D spectra the three intense signals were definitely assigned and they are intended for the substituted group at 6-position on the pterin ring. As mentioned by 2D-NMR spectra of natural substance (see figure 3.14) here it was shown that C-11 carbon nuclei at δ = 19.6 ppm was coupled to directly bonded methyl proton at δ = 1.08 ppm, C-10 (β)-carbon at δ = 67.9 ppm to the proton bonded hydroxyl group at δ = 4.0 ppm and C-9 (α)- carbon at δ =73.7 ppm to the proton bonded hydroxyl group at δ = 4.45 ppm respectively. These signals had very similar chemical shifts to those of the natural substance. Based on comparing the (^{1}H, ^{13}C)-COSY data, this isolated system could be assigned to the structure-



The conclusion from comparison of all the NMR results was in agreement for containing the 7-hydroxypterin in natural substance. Furthermore, it is a suitable model compound for the biologically active sample.

^{1}H NMR, ^{13}C NMR and (^{1}H, ^{13}C)-COSY spectra of standard 2 are shown in appendix B2.1, B2.2 and B2.3 (page 122-124). Key features in ^{1}H NMR were a doublet at δ =1.22 ppm and a multiplet near δ 5.3 ppm and these signals were easily assigned to the structure >CH-CH_3, which, as a substituted group, could be attributed to the 6 position of isoxanthopterin ring. By comparing these two signals with those of natural compound, a doublet at 1.22 ppm had similar parameter, however the position of a multiplet at 5.3 ppm was not similar to that of natural sample (see figure 3.11, page 68).

The ^{13}C NMR spectrum of standard 2 (page 123) also showed chemical shift at 70 ppm for the carbon of α-CH-OH group and at 21 ppm for the carbon of CH_3 group. Comparing the ^{13}C NMR spectrum of standard 2 with the spectrum of the corresponding active sample, the chemical shift of CH_3 carbon was

identical while the position of α-carbon was not identical. However, from the NMR data it is not possible to verify that the biologically active substance is of standard 2 compound.

Laser desorption ionization mass spectra of standard compounds 1 and 2 are illustrated in appendix C1 and C2 (see page 126-129). In accordance with the comparison of LDIMS corresponding to the authentic compounds and natural substance more detailed structural information may be inspected. In positive ion, mass spectrum of standard 1 (see page 126) consisted of pseudo-molecular ion peaks at m/z 254, 276, 292 and 298 which were directly related to $[M+H]^+$, $[M+Na]^+$, $[M+K]^+$ and $[M+2Na-H]^+$ ions respectively. In addition to these ions the important peak at m/z 180 was accounted for in this spectrum and it represented the loss of substituted group at the 6 position on the heterocyclic pterin. Compared with the ion fragments in unknown spectrum of active sample (see page 73) they were very similar. These overlaps confirm the molecular weight of active sample proposed to 253 previously. As could be seen in appendix C1.2 (page 127) negative ion mode of standard 1 was detected to the deprotonated analyte ions at m/z 252, 208, 206 and 178 due to the ion fragments. As a result, in comparison of mass spectra of standard 1 and active sample they showed the identical ions which were of major significant peaks in deducing the molecular structure (see chapter 3.2.4). This clearly shows that standard 1 is an active sample.

Examining the positive-and negative-ion spectra of standard 2 (see page 128 and 129), the important peaks could be seen at m/z 224 $(M+H)^+$, 246 $(M+K)^+$, 262 $(M+Na)^+$, 268 $(M+2K-H)^+$, 206 $(224-H_2O)^+$ and 178 $(224-CH_3CHO)^+$ for positive mode and 222 $(M-H)^-$, 178 $(222-CH_3CHO)^-$ and 135 $(178-CONH)^-$ for negative ions respectively. However, only a peak at m/z 224 was found in positive mode of active sample (see page 73) and the others were not clearly detected. In comparing the negative spectra of standard 2 and active sample the important negative ion at m/z 222 was not present for the active sample.
Nevertheless, from the result of mass spectra, it should be concluded that the active sample is not easily identified in the standard 2 compound.

3.2.8 Summary of pheromone identification

With the LDI mass spectrometric results, an $(M+H)^+$ ion (see figure 3.15, page 73) was discovered indicating a molecular weight of 253 which would fit the elemental composition of $C_9H_{11}N_5O_4$ of the proposed 7-hydroxybiopterin. UV spectroscopic analysis of isolated active sample proved the dependence on the pH values of solvents and this result could support the hypothesis, which deals with the dissociated substance. As previously mentioned, UV spectrometric considerations for the substituted group of active sample led to the conclusion that it was bonded by an isoxanthopterin compound. The agreement of spectral NMR data of the skin extracted substance and an authentic standard 1 led us to propose an alarm substance, which had the same structure as the standard 1. According to the structural hypothesis by UV measurement, a postulated molecular mass by mass spectroscopy and detailed structure identification by NMR, the alarm pheromone extracted from skin of *Danio malabaricus* was finally found to be the pterin structure 7-hydroxybiopterin. The results of chromatographic, spectroscopic, analytical and physiological behavioral investigations with the alarm substance and synthesized 7-hydroxybiopterin were in total agreement.

3.3 Identification of fraction G1 from GFC

The fraction G1, co-eluted together with the pheromone substance in the third chromatographic step, was successfully separated from the pheromone mixture by the GFC method (see chapter 3.1.3.4.2). Through pheromone isolation from the skin extract about 3 mg of this substance was achieved. The sample was completely dissolved in 600 µL D_2O and subjected to ^1H NMR-studies. The ^1H NMR spectrum of fraction G1 is given in appendix D1 (see page 131). In this spectrum of G1, the two singlets near 8 ppm and a doublet at 6.1 ppm and a cluster of signals between 4 and 5 ppm were found. One of these singlets was intense and the other was too small. From the comparison of reference spectra, these two singlets >8 ppm could be the protons H(8) and H(2) of a hypoxanthine or adenine structure. Comparison of the hypoxanthine and adenine spectra with the spectra of the corresponding nucleosides explained the doublet at 6.1 ppm. The signals between 4 and 5 ppm were assigned to

the ribose protons H(2) and H(3). The signals at 3.8 ppm were due to the methylene proton H(5) of ribose. Based upon these data substance in fraction G1 from GFC could be postulated as inosine or adenosine (see figure 3.18). However, by comparison with the UV spectra of these substances and natural substance in fraction G1, it was clearly identified as inosine (see table 3.17) (appendix A4.1-A4.2, page 117).

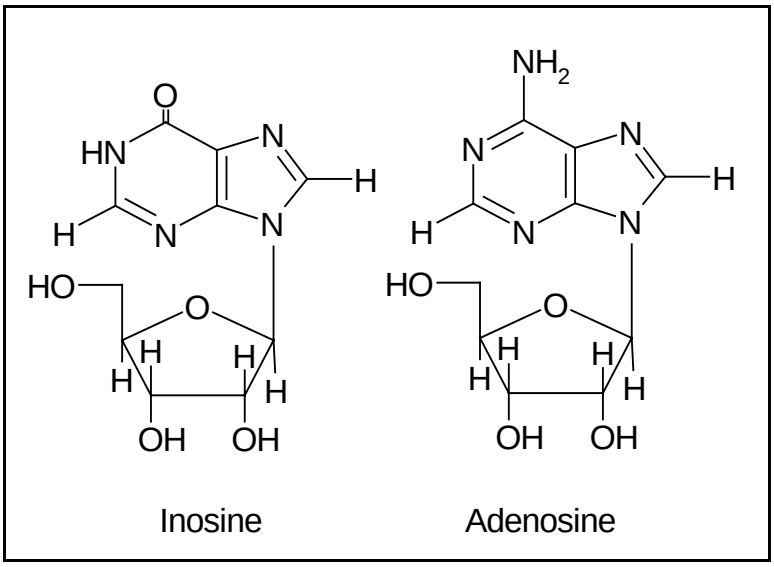


Figure 3.18. Structures of inosine and adenosine

Substance	λ_{max} (nm)	λ_{max} (nm)	Solvent
adenosine	205	258	water
inosine	198.2	248.6	water
fraction G 1	198.2	248.6	water

Table 3.17. UV data of adenosine, inosine and fraction G 1

The assignment was verified by using the ^{1}H NMR measurement of pure inosine ($C_{10}H_{12}N_4O_5$, MW 286.23 g/mol, appendix D1, page 132) in D_2O and by comparing the retention time in RP-HPLC chromatogram.

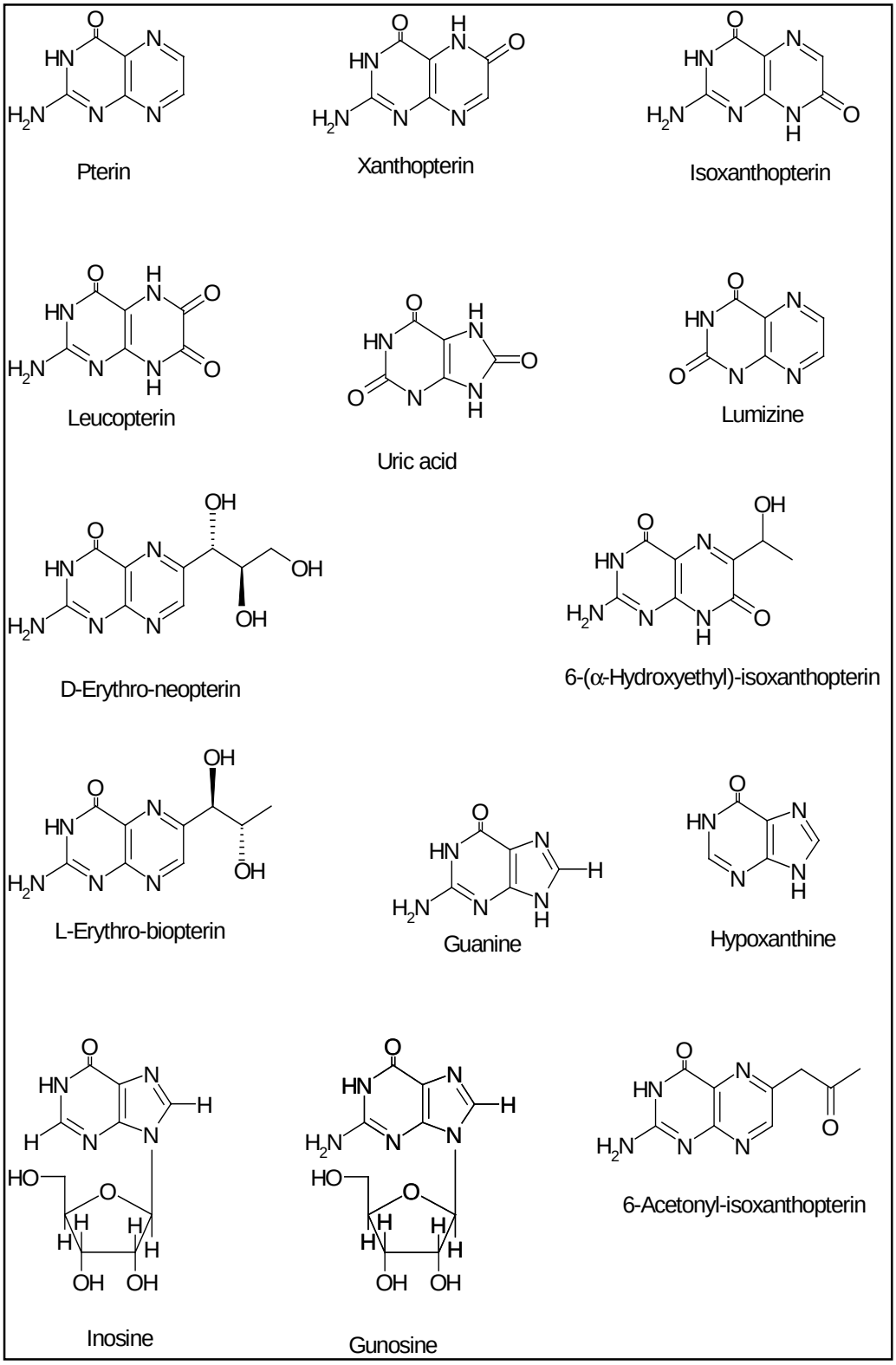
3.4 Test-results of other substances

Based on the structural formula of 7-hydroxypterin, experiments were done to determine the effect of the related structure.

The objective of this experiment was to bioassay for the specific and selective pheromonal effects of these structures. These experiments were to carry out the qualitative behavioral bioassays with the substances, which were selected under certain criteria. Conclusions were then drawn from the bioassay results of the different type of reactions due to the effects of these structures. The following different selected criteria were considered for tested substances.

- These substances should contain the fragments of 7-hydroxybiopterin or should either be a variation of the functional groups of it or other substitutions of isoxanthopterin. Apart from the substitution, certain substances that are excluded from the functional groups were examined in biotest.
- These substances should be obtained from purine derivatives or pyrimidine derivatives.
- All substances should be dissolved in water solvent and the amounts tested should be mostly the same.
- Most of the substances should be obtained commercially.
- All substances should possess a high purity grade.

The structures of the selected substances are described as follows:


Pterin
Xanthopterin
Isoxanthopterin
Leucopterin
Uric acid
Lumizine
D-Erythro-neopterin
6-(α-Hydroxyethyl)-isoxanthopterin
L-Erythro-biopterin
Guanine
Hypoxanthine
Inosine
Gunosine
6-Acetonyl-isoxanthopterin

The concentration statements (in water), the test volumes, bioassay results and literature references are described in table 3.18.

Substance	Conc. ($mol \cdot L^{-1}$)	Test number	Vol. (mL)	Bioassay-result	Reference
pterin	6.13×10^{-4}	2	15	$(- -)^2$	Merck-chemical catalog (1997/98)
leucopterin	5.13×10^{-5}	3	25	$(- -)^3$	Fluka-chemical catalog (1999)
biopterin	3.95×10^{-5}	3	25	$(- -)^3$	Fluka-chemical catalog (1999)
neopterin	3.95×10^{-5}	4	25	$(- -)^3$ $(+ -)^1$	Fluka-chemical catalog (1999)
lumizine	3.05×10^{-4}	3	20	$(- -)^3$	Merck-chemical Catalog (1995)
uric acid	5.95×10^{-4}	3	20	$(- -)^3$	Merck-chemical Catalog (1995)
xanthine	3.13×10^{-4}	3	20	$(- -)^3$	Merck-chemical Catalog (1995)
hypoxanthine	3.68×10^{-4}	3	20	$(- -)^3$	Merck-chemical Catalog (1995)
inosine	3.73×10^{-4}	4	20	$(- -)^4$	Merck-chemical Catalog (1995)
gunosine	3.50×10^{-4}	3	20	$(- -)^3$	Merck-chemical Catalog (1995)
xanthopterin	5.07×10^{-5}	4	20	$(- -)^3$ $(+ -)^1$	Fluka-chemical catalog (1997)

Substance	Conc. ($mol \cdot L^{-1}$)	Test number	Vol. (mL)	Bioassay-result	Reference
6-(α-hydroxyethyl) isoxantho-pterin	4.48×10^{-5}	4	25	$(+ +)^4$	Sprison (1946) & Tschesche and Glaser (1958)
isoxantho pterin	5.07×10^{-5}	4	20	$(+ +)^4$	Fluka-chemical catalog (1997)
6-acetonyl isoxantho-pterin	4.25×10^{-5}	4	25	$(+ +)^4$	Tschesche and Glaser (1958)

(– –): negative reaction; (+ –): questionable reaction; (+ +): positive reaction; super-script of bracket: numbers of tests

Table 3.18. Bioassay results of related substances

According to table 3.18, only three substances clearly showed the positive fright reaction in the bioassay. The activities of these substances were very closely identical. However, these substances reacted when concentrated 5 times more than the 7-hydroxybiopterin. All the other substances did not show the fright reaction. 7-hydroxybiopterin structure was therefore the high specific pheromone structure. Only the structure consisting of isoxanthopterin body was able to show the activities. Without this main compound it could lose the activity.

4 Discussion

Chemical intraspecific and interspecific regulation of fish behaviour is performed by specific products. One such chemical signal, shaping the escape behaviour in danio fish is the alarm pheromone. A substance, which is excreted from the skin of the fish when this is damaged, evokes a fright reaction in individuals of the same species.

Although both, the alarm substance containing cell and fright reaction in ostariophysi fish are well known, a detailed chemical analysis of the pheromones has not been performed. Scientists have only proposed that the alarm substance was likely to be a purine- or pteridine- substance. In order to prepare a maximum amount of the alarm pheromone containing material, it was extracted from the skin of fish using the methods described in chapter 2.5. To isolate the pheromone from this skin extract, two different basic methods were applied:

- After using a mortar and homogeniser, the skin extract was diluted with millipore water and subsequently centrifuged (method 3 in chapter 2.5).
- In addition to homogenisation, the diluted aqueous homogenate was extracted with diluted 10% acetic acid and then again diluted 5% acetic acid. For a complete separation of the alarm substance from solid constituent, it was finally used by centrifugation (method 4 in chapter 2.5).

Both of these methods were used in this work. However, in order to obtain the quantitative isolated pheromone, the optimal methods had to be selected. As mentioned in chapters 2.5 and 3.1.1, the first method was suitable for a water-soluble substance. It was difficult to isolate a large amount of the signal substance by extraction with water. Therefore, the second method was selected for the preparation of a pheromone-containing sample. Although the procedure for a manual removal of the skin requires some time, it was impossible to homogenise the whole body of fish. This procedure would be unfavourable in

the variation of the quality and quantity of pheromone containing materials because of the other biologically inactive compounds of the body of fish, which are near the skin extract. These inactive dissolved compounds are expected to be difficult to isolate the alarm material from the complex mixture. Ultrafiltration was used in the final separation. This procedure was a very suitable process not only to cut off the molecular weight of compound mixture of interest but also to isolate the sample for further separation steps (see chapter 3.1.2.2).

To isolate the alarm pheromone from this complex mixture, the selection of chromatographic methods was adjusted after the respective variation of skin extract. For the first chromatographic process, reversed-phase high-performance liquid chromatography (RP-HPLC) was selected. As mentioned in chapters 2.9.3 and 3.1.3, using the nonpolar stationary phase and a polar mobile phase, the biologically active compound was successfully separated from the complex mixture. A pheromone sample with low water solubility was fractionated in the region of methanol gradient to obtain the polar and non-volatile compound. The shortest and most effective way for isolating and concentrating the alarm pheromone was realised by the three consecutive chromatographic steps. In the first HPLC step, by the methanol equilibrium of the separation phase, the active fraction resulted at the reversed phase within a short time. This was favoured to elute the polar compounds with a short retention time (see chapter 3.1.3.2). Besides, it was possible to separate the biologically inactive compounds with a longer retention time. Rechromatography of biologically active fractions improved separation. In the second separation step, the active fraction with a longer retention time than that in the second step was eluted by changing the flow rate. To obtain a chromatographically pure substance, the active sample fraction was co-eluted for a third separation step. In this way, pheromone-containing fraction was isolated from the skin extract mixture as a single-peak fraction (see chapter 3.1.3.4). However, the chromatographically pure pheromone substance could not be obtained by RP-HPLC, therefore, another way was considered to obtain a good separation of the pheromonal sample from the mixture component. As described above, an excellent isolation was achieved by gel filtration chromatography (GFC). In this case, by using the Toyopearl HW40-S gel, a single peak deducing from 3. HPLC step was entirely separated to gain two different peaks (see figure 3.5). This result is evidenced here as the best way because this major compound obtained could not be derived by reversed phase. Therewith the impurity

longer retention time and higher molecular weight than pheromone component could be removed. Under this condition, it was important to select the available separation parameter and a suitable stationary phase. Using GFC it seems that a “chromatographic chemical pure” substance was isolated. Indeed, it was absolutely necessary to check the purity of isolated biologically active fraction. To clarify this, the purity of active fraction was examined by using the other waters instrument with photodiode array detector (PDA), new chromatographic column, other systems and thin layer chromatography (see chapter 3.1.3.6). Finally, these results showed that “spectroscopic chromatographic pure” substance could be elicited. It was impossible to determine the structure of pheromone only by UV spectroscopy. Although the characteristic UV absorption spectra of pheromones (see chapter 3.2.1) by varying the pH values of solvents were not achieved to create the structural hypothesis of the pheromone, the result of this experiment showed the dissociated character of pheromone substance by using the variation of pH values of solvents. As for chapter 3.2.2, it should be concluded that the alarm substance was not a purine derivative compound. However, according to the comparisons of some pterin compounds as the chemical pre-information related to pheromone, the structural hypothesis of it could be investigated as well one of the isoxanthopterin derivatives.

With regard to the UV spectroscopic determination, detailed structure information could not directly be obtained. To solve this problem, ^{1}H and ^{13}C NMR measurements were selected. Using the deuterated DMSO solvent, the substance was fully dissolved to measure NMR spectra. It was not possible to use the D_2O solvent because of low solubility of active substance in water. ^{13}C NMR was suited to identify the structural analysis of unknown compound. The NMR results from chapter 3.2.3 showed that bioactive substance was postulated as 7-hydroxybiopterin. Indeed, this identified structure concerning the probable ^{1}H, ^{13}C, 2-D (^{1}H-^{13}C-COSY) NMR spectra required to compare with the authentic samples, conclusive evidence for the existence of proposed compound. In order to compare with the NMR spectra, authentic compounds was synthesised (see chapters 3.4 and 3.5). After this, from comparison of all the NMR measurements it can be concluded that the 7-hydroxybiopterin, which is a model compound for the natural alarm substance. Perhaps, as proposed above, this structural elucidation of the NMR analysis was confirmed by using the LD-MS spectra (see chapter 3.2.4). From the results of LDIMS

spectra, it was possible to postulate accurately that the molecular mass of the alarm substance is 253 $g \cdot mol^{-1}$. Basically, the mass spectroscopic comparison of postulated pheromone structure with the authentic synthetic material was carried out. In this way, pheromone was identified as 7-hydroxybiopterin. Conclusive evidence for corresponding structure of natural alarm and synthetic substance was equivalent to UV spectroscopic data (see chapter 3.2.2).

The second unknown compound found in the NMR spectra could be a pterin, probably chemically related to 7-hydroxybiopterin, was not a 6-(α-hydroxyethyl)-isoxanthopterin (see chapter 3.5), may be 2 carbon side chain at the 6 position of isoxanthopterin ring. From the ratio of NMR spectral data, by comparing the main compound named 7-hydroxybiopterin with this unknown compound its constitution found was smaller than that main compound. Although the consideration of others closely related 7-hydroxybiopterin compound, the NMR data of the postulated compounds differed from that obtained from the natural sample. In this case, it was very difficult to confirm the identity of the isolated natural and the proposed compound. In order to elucidate how these compounds are isolated, some separation methods are needed. It could be considered that high-performance liquid chromatography and gel filtration chromatography may be used for separation. However, there were also insufficient methods available to separate the major compound and the second unknown compound with certainty, because these compounds are very closely related to each other.

With the variation of pH values of solvent, the UV spectra of the pheromone pointed to the characteristic shifting of absorption maximum (see figure 3.9). It is important to define the basic UV spectrum at the different molecular forms and to know the dissociation sequence of acidic hydrogen atoms in diluted two basic 7-hydroxybiopterin. According to Pfleiderer (1985), the dissociation sequence of acidic H-atom predicted to be N^8-hydrogen greater acidic than N^3-hydrogen. The structure of three molecular forms of neutral molecule, mono- and dianions with the respective characteristic UV absorption spectroscopic data are all together shown in figure 4.1.

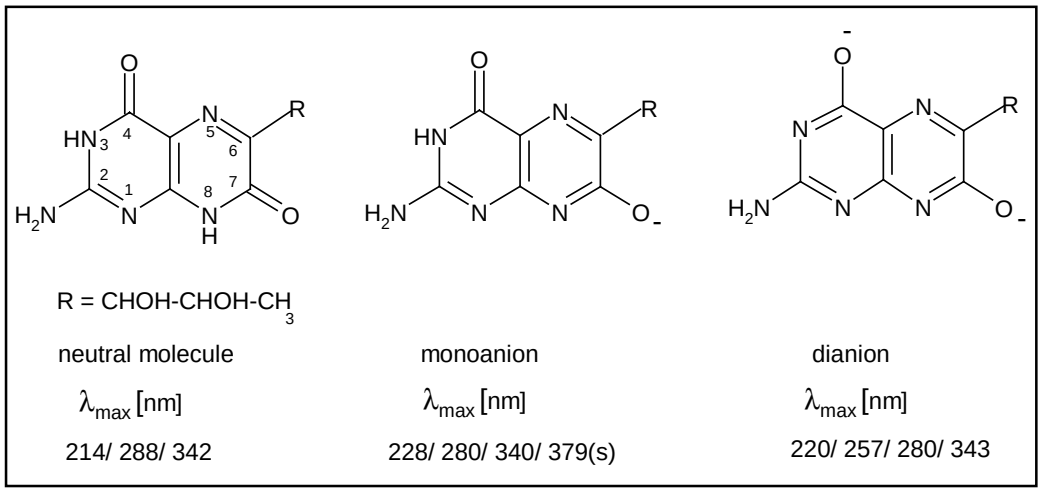


Figure 4.1. Dissociation of 7-hydroxybiopterin and UV-maximum

7-hydroxypterin does not have a characteristic melting point and decomposes at about 300°C. It is soluble in alkali but almost insoluble in neutral or acidic media and common organic solvent (Nawa et al., 1954).

7-hydroxybiopterin in water, which is stored at –25°C, is still bioactive to 8 months. Solubility of 7-hydroxybiopterin in water is less than that of biopterin whereas the presence of more than one hydroxy group on a nitrogen-containing heterocyclic ring and is more than that of isoxanthopterin whereas the presence of three carbon side chain at the 6^{th} position of pterin ring. Isoxanthopterin shows also low solubility in water, requiring 200 L of water to dissolve 1 g at room temperature. The solubility of 7-hydroxybiopterin in water or diluted HCl is about 0.3 mg mL^{-1}. According to Pfleiderer (1985), one hydroxy group decreases solubility by a factor of 28- to 500-fold, and introducing of more hydroxy groups further decreases the solubility gradually depending upon the number and position of the functional group. The presence of both amino groups and hydroxy groups in 7-hydroxybiopterin decrease the solubility tremendously. These hydrophilic groups exert even more attraction to one another than they do for the molecules of water. It should be realized that the anion (see figure 4.1) and cation of 7-hydroxybiopterin are as ionic species considerably more soluble.

According to Hüttel et al. (1943), ichthyopterin as a violet-fluorescing natural substance was firstly isolated from the skin of carp fish. This isolated ichthyopterin compound is pH dependent and has soluble properties. The solubility of it in water or diluted HCl is about 0.3 mg·mL^{-1}. Exact chemical proper-

ties of ichthyopterin are still unknown. Thirty years ago, it was postulated that ichthyopterin is a major component of the pteridines in the skin and scales of cyprinid fish and this compound was demonstrated to be 7-hydroxybiopterin (Tschesche and Glaser, 1958). The configuration of hydroxyl groups on the side chain remain to be determined (Hüttel et al., 1943; Tschesche and Glaser, 1958 and Kauffmann, 1959). Hama et al. (1960) and Ohta et al. (1968) reported the occurrence of an enzyme, which catalyses 7-hydroxybiopterin formation from Cyprino-purple C in cyprinid fish. Cyprino-purple C is another violet-fluorescing substance contained only in the skin of fry. The chemical structure of this compound is also 7-hydroxy-6-substituted pteridine. Therefore, the enzymatic conversion from it implies the alteration of the side chain at position 6 on the pterin ring, and it is as yet not still clear in which step the position 7 of the pterin ring is oxidised during the course of the biosynthesis of 7-hydroxybiopterin. With regard of the biosynthesis of tetrahydrobiopterin, it was investigated that an occurrence of the enzyme which is contained in fish, is something like xanthine oxidase (XO) or xanthine dehydrogenase (XD) (Takikawa et al., 1986). These enzymes catalyse the oxidation of pterin into isoxanthopterin, and xanthopterin into leucopterin. In view of the biosynthesis of 7-hydroxybiopterin (Takikawa et al., 1989), biopterin was used as a substrate for the new enzyme, because formation of 7-hydroxybiopterin from biopterin (L-erythro-biopterin) is only one step reaction. They reported that this new enzyme which is different from XO and XD, is very unstable and the activity in an elute diminished to 20% after being kept at 4°C for 2 days. The biosynthetic pathway of biopterin is shown in figure 4.2 (http://www.unizh.ch/~blau/-pterins/catabol.gif, Ziegler, 1987 and Takikawa et al., 1989). The probable pathway for a conversion of 7-hydroxybiopterin into isoxanthopterin is also shown in figure 4.2. It is possible that the three-carbon side chain from 7-hydroxybiopterin is released by non-enzymatic process. But, it's non enzymatic conversion is not as yet clear and one possible is to cleave the side chain by reduction of Al-Hg in alkaline media (Matsuura et al., 1955). No other case has been reported for the formation of isoxanthopterin except by enzymatic conversion of the corresponding 7-hydroxybiopterin.

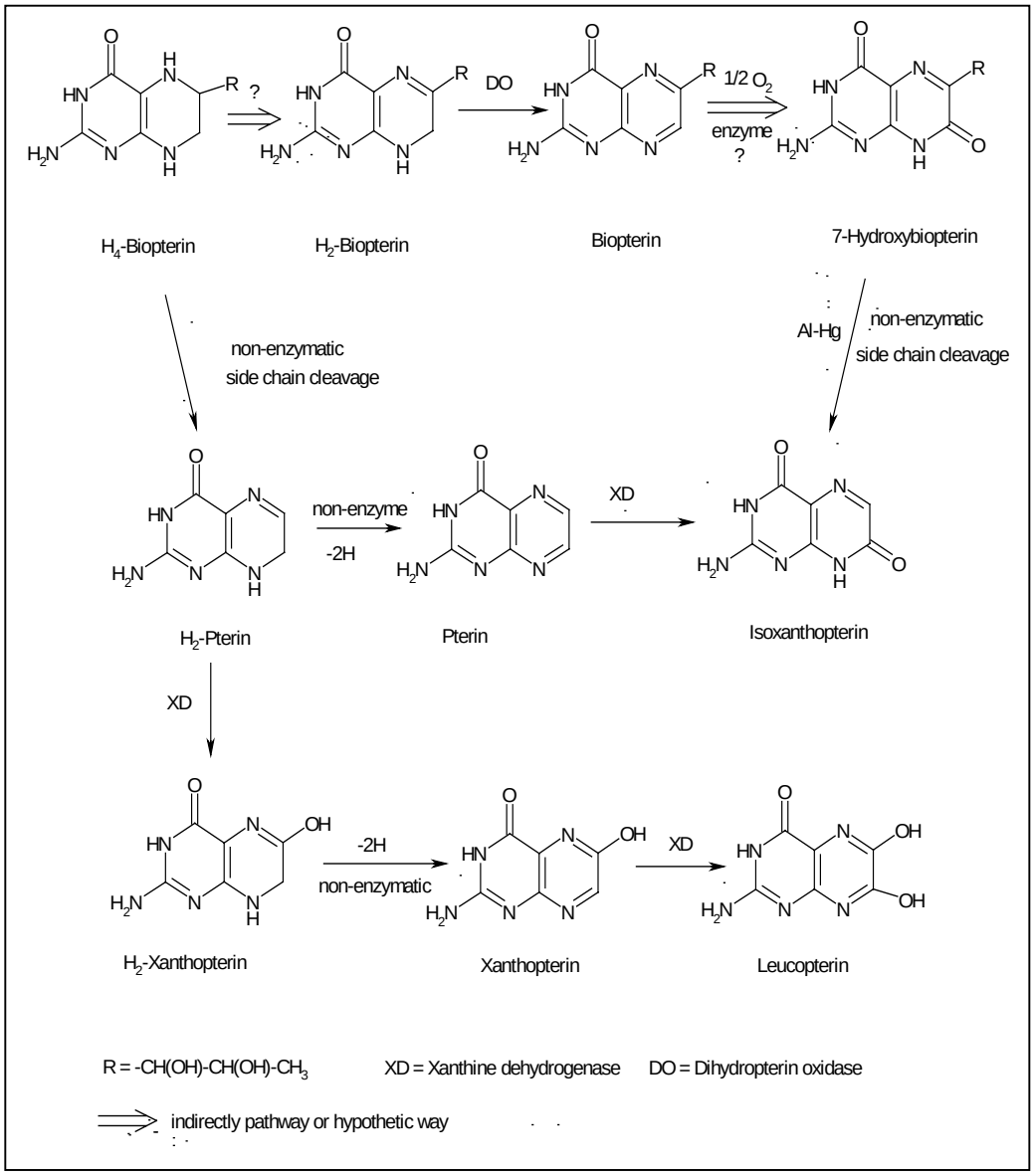


Figure 4.2. Probable bio-pathways of biopterin (from Ziegler, 1987; Takikawa et al., 1989 and Blau [internet])

From the structure of 7-hydroxybiopterin, it can be seen that the side chain of the pterin ring contains two chiral centers. However, the configuration of 7-hydroxybiopterin for dihydroxypropyl groups on the 6-position of the isoxanthopterin ring is not well known. This pterin is considered to designate the L-, D-erythro or L-, D-threo side chain configurations or mixture of them. Possible structures for two chiral carbons in side chain respresented as two pairs of diastereomers are shown as follows.

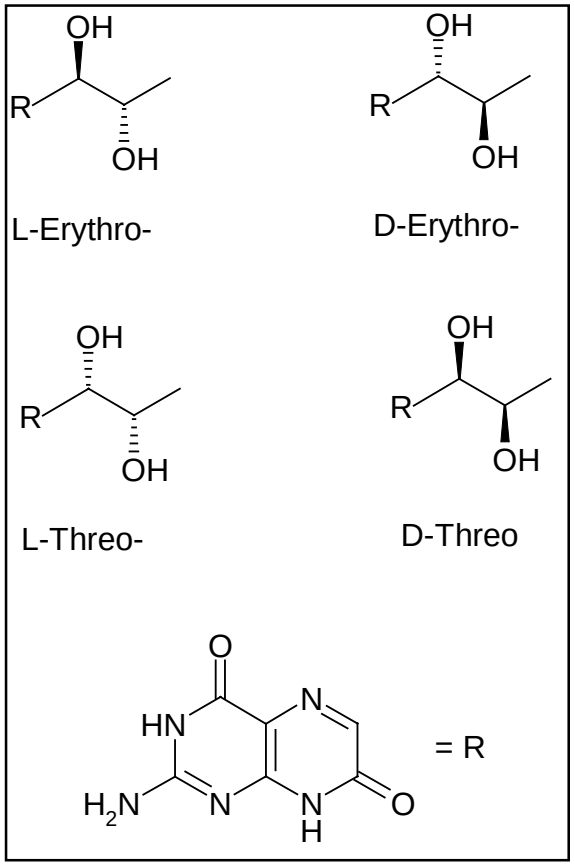


Figure 4.3. Configurations of diastereomers

According to the data of ^{1}H and ^{13}C NMR spectra, α carbon (C-9) of side chain was observed only as doublet and two doublets were not found in all NMR spectra. This could be effected to interpret that the configuration of 7-hydroxy-biopterin could not be mixed isomers. It should be only one diastereomers.

Another well-known pterin that contains a three-carbon side chain is biopterin [L-erythro-6-(α,β-dihydroxypropyl)-pterin], shown in figure 4.4 (Brown, 1985).

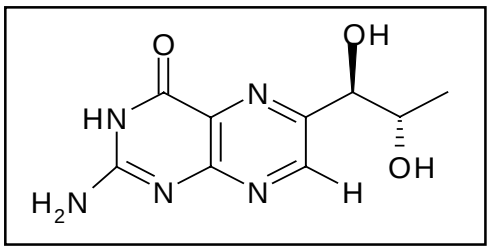


Figure 4.4. Structure of L-erythro-bio-pterin

This configuration was confirmed by comparison with the synthetic isomer which was made by condensation of 2,4,6-triaminopyrimidin-4-one with 5-deoxy-L-arabinose.

In the biosynthesis of H_2biopterin and sepiapterin (Brown, 1985), sepiapterin could be reduced directly to H_2biopterin in the presence of NADPH (pyridine nucleotide) and then biopterin was oxidized from H_2biopterin by an enzyme dihydropterin oxidase. This biosynthesis is precisely identified that the configuration of biopterin is L-erythro.

Takikawa et al. (1989) postulated that 7 position of biopterin [L-erythro-6-(α,β-dihydroxypropyl)-pterin] oxidizes to the 7-hydroxybiopterin by an enzyme (see figure 4.2). Concerning this investigation, the most likely possibility is that the configuration of side chain at the 6 position of 7-hydroxybiopterin is an L-erythro group (see figure 4.5).

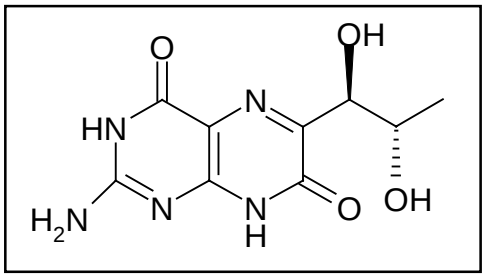


Figure 4.5. Structure of L-erythro-7-hydroxybiopterin

Nakagoshi et al. (1982) reported the fluorescent spectrum of the 7-hydroxybiopterin using an excitation wavelength of 348 nm and isoxanthopterin-6-carboxylic acid formation by alkaline potassium permanganate oxidation of 7-hydroxybiopterin. In this case, the side chain of 7-hydroxybiopterin converted to the carboxylic group. 7-hydroxybiopterin, isoxanthopterin-6-carboxylic acid have the strong fluorescent characters. 6-carboxyisoxanthopterin is probable degradation product of the 7-hydroxybiopterin. Thus, oxidized 7-hydroxybiopterin such as 6-carboxyisoxanthopterin is also present in the skin of fish. In RP-HPLC chromatograms, it could be seen that there is a slight peak at about 3 minutes (retention time). It has no characteristic absorption band if the chromatograms were measured at wavelength 200 nm. It could be estimated that this weak peak which is a degraded product of alarm substance (proposed as 7-hydroxybiopterin) could be 6-carboxyisoxanthopterin (see figure 4.6).

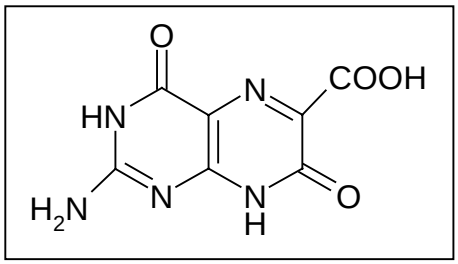


Figure 4.6. Structure of 6-carboxy-isoxanthopterin

Hall et al. (1995) proposed that fright reactions occur in response to alarm substance passively released by injury to the skin of zebra danio fish (*Brachydanio rerio*). Their fish are experimentally naive. Visual observation of alarmed conspecifics yields socially facilitated alarm by observers. Concurrent exposure of fish to alarm substance and a novel chemosensory stimulus can transform the novel chemosensory stimulus into a learned elicitor of fright behaviour for zebra fish. Visual transmission and social facilitation of fright behaviour can result in a very rapid spread of alarm behaviour to all fish in visual contact. In order to support this result, the responses of adult zebra danio fish (*Brachydanio rerio*), which were unlearnt fright reaction, in aquarium to alarm substance extracted from the skin of conspecifics were analyzed. The fish´s response was detected by using the video camera. After introducing the extracted alarm substance, they did not elicit the fright reaction and no other response. We know that the intensity of the response depends on the pheromone amount. In this case, although 3 times greater than normal pheromone amount was used, no fright reaction was evident. Results on the development of the alarm response indicate that zebra fish did not react to the alarm substance in our laboratory. Von Frisch (1941b) reported that there is evidently a strong reaction in the alarm substance within the Ostariophysi and the Gonorhynchiforms.

Again, the other possible bioassay was examined by the introduction of a solution extracted from a surgically damaged zebra fish into a school of individuals of same species. The school was not frightened. Von Frisch (1938) accidentally discovered the fright reaction from an observation while experimenting with the European minnow. This was associated with the introduction of a surgically operated minnow into a school of conspecifics. Von Frisch had marked one fish by severing the Nervus sympathicus near the tail, thus producing a darkening of the skin caudal to the incision. When the fish was intro-

duced into the normal school, some of the individuals retreated rapidly and the school was obviously frightened. With the knowledge of this experiment, a damaged zebra fish was introduced into the school of individual conspecifics. They crowded together slightly, but the reaction immediately disappeared and the school showed the normal behaviour. Furthermore, as the last test for zebra fish, killed zebra fish was suspended in a test school. Unfortunately, they happened not only nothing further fright response but also feeding behaviour. Pfeiffer (1963a) found that young zebra fish (20 days old) contained alarm substance in their skin and that this skin produced a fright reaction in adult fish of the same species prior to the first reaction in the young zebra fish.

Dealing with this subject it is important to consider that adult zebra fish, which present the alarm substance cells in their epidermis, but could not smell the alarm substance or these zebra fish contain undeveloped alarm cells in the skin. But why- the zebra fish did not react to skin extract or damaged skin of conspecifics?

In order to understand this problem we should get a better understanding of the alarm signal system in fish and distribution of their fright reaction.

Until now, there has been no extensive study of the alarm reactions and chemical structure identification of natural alarm substance in zebra danio fish.

In order to prove whether an alarm substance really exists in fish skin or not, it can generate in fright response associated with conspecifics or not, fish react to the authentic alarm substance which is the chemically same structure with the natural alarm substance or not, the most interesting species of Ostariophysi, giant danio, *Danio malabaricus,* which presents the best-understood function of alarm reaction, was observed. It is one part of my objective and another important aim is to postulate the pheromone´s chemical structure of giant danio by using analytical methods.

The chemical nature of the alarm substance from the skin of the giant danio, *Danio malabaricus*, has not yet been identified, in spite of the investigation of the alarm structure from the skin of minnow by Argentini (see chapter 1.4). By using the preparative and analytical methods, we obtained the result indicating that the main compound of the alarm pheromone is of 7-hydroxybiopterin a pterin substance, and does not consist of a purine. These findings do not correlate with the results of Argentini (1976). As described in chapter 3.3, the inactive fraction (G1) found in GFC was successfully identified as a ribonucleoside inosine component by ^{1}H NMR spectroscopy. In this case, inosine did not show the alarm response. Therefore, inosine was not an important com-

ponent for this work. Interestingly, Argentini´s result may be compared with this inactive inosine. It is possible to convert hypoxanthine from inosine by enzymatic transformation. In this condition, to leave the ribose it needs the nucleosidase catalyst. As a rule, therefore, in natural condition, inosine is difficult to change to hypoxanthine without nucleosidase. In the case of hypoxanthine, oxidation at room temperature over a three-month period produced only small yields of an oxidation product, but decompose when an attempt is made to isolate them. Most of the purine N-oxides is quite stable in natural aqueous solutions over a long period and there is no tendency for the oxide to lose the oxygen and revert to purine (Stevens et al., 1958).

Argentini (1976) reported that hypoxanthine was also ineffective in the behavioural experiments with fish schools. Only hypoxanthine-3(N)-oxide found for the full biological activity. He concluded that the instability of hypoxanthine-3(N)-oxide fits with the assumption that this substance is identical with the unstable alarm substance. According to Stevens et al, hot acetic acid may convert the N-oxide to an unidentified material resembling hypoxanthine. Under the natural conditions, N-oxide could be stable. Since the alarm substance in water was stable only for 26 hours, these two possibilities are not likely.

Furthermore, according to von Frisch (1941b), the alarm pheromone is responsible for three days at 16°C and it is not possible to destroy it in natural conditions. His finding corresponds to our experiments in which alarm substance extracted from danio skin was kept for 68 hours at room temperature and it was as effective as fresh skin in eliciting the fright reaction. There was no difference in the fright reaction between an aged sample (68 hours) and a freshly prepared skin extract. Moreover, the efficiency of alarm substance was also observed in some experiments. The diluted skin extracts from danio fish heating about 50°C for 30 minutes did not reduce its effectiveness to the fright reaction by skin extract without heating. From these experiments, it could be suggested that the alarm substance is stable under the natural conditions and can not be immediately destroyed. At the same condition, 7-hydroxybiopterin has also shown the identical properties of the alarm substance. This result supported the hypothesis that the alarm substance from the skin of *Danio malabaricus* is identical with 7-hydroxybiopterin.

According to bioassays with an authentic alarm substance (7-hydroxybiopterin), the amount of substance solutions more than 5 mL tested elicited the fright reactions. It can be concluded that bioassays of amount less than 5 mL induced the questionable reactions and negative reactions.

Additionally, the bioassays with the some different compounds concerning the purines and pterins derivatives were performed to check the pheromonal activities on the danio fish (see chapter 3.6). This study also concluded that purine derivatives led to a loss of biological activity, e.g., xanthopterin, inosine and gaunosine induce no response in danio. According to these experiments, the only three-pterin derivatives, which are isoxanthopterin, 6-carboxylisoxanthopterin and 6-(α-hydroxyethyl)-isoxanthopterin, elicit the fright reaction. However, these behavioural reactions were investigated by using the amount of related compounds of 5 times more than that of 7-hydroxybiopterin. On the contrary, isomer of isoxanthopterin, xanthopterin as well as the other pteridines tested was not clearly effective in the bioassay. It could be concluded that, the basic structure including isoxanthopterin probably demonstrated an alarm reaction. Without this chemically basic structure it could lose its biological activity (see chapter 3.6).

From these results in chapter 3.6, it seemed to agree to what has been done by Pfeiffer (1978) and to argue against the work of Argentini (1976) who postulated that a purine derivative was found as the alarm substance.

At present, all our results examined in bioassays were qualitative, but not quantitative. However, Pfeiffer et al. (1985) proposed the quantitative study of alarm response using the black tetra, *Gymnocorymbus ternetzi*. hypoxanthine-3(N)-oxide was found to be as effective as skin extract in producing the behavioural response. In their work, a videorecorder was used to measure the change in the angle of inclination of the dorsoventral axis of the fish. They concluded that this substance, hypoxanthine-3(N)-oxide, which was identified by Argentini, as the alarm pheromone is the active compound. After this work, Pfeiffer did not make any publication concerning the quantitative measurement of the alarm response.

The variety of life histories and biological adaptations in the fishes, combined with the potential of several different, independently evolved alarm signals should provide many avenues of approach and potential research subjects for examining the evolution of alarm systems. There have been many interesting effects reported in other groups of animals that many occur in fish and which would extend both the biological interest of these systems and their generality. The morphological and life cycle responses were found in vertebrates. Birds show deceitful alarm signalling, in which senders give false alarm calls to distract receivers from food or other resources. Audience effects occur in domestic chickens, they are more likely to give an alarm call if with a companion than

when alone. Vervet monkeys assess the reliability of individual signallers and tend to ignore signals from untrustworthy individual predators that prey upon individuals, in contrast to other individuals of the same predatory species that do not.

The many effects of alarm signalling that have been documented or proposed in fish or other organisms indicate that this phenomenon must be taken into account in any examination of foraging tactics or predator-prey interaction or any of the several areas of decision making that could be influenced by information on predation risk. Alarm signaling is much more widespread than was previously thought (Smith, 1992).

Alarm pheromones have been described from several fish orders but details are only known for the Ostariophysi and the Gonorhynchiformes. In the superorder Ostariophysi, specialised epidermal cells contain the alarm pheromone and these cells have no other known function (Pfeiffer, 1960, 1977). Chemical alarm pheromones, or Schreckstoffe, of fish in the Ostariophysi, which includes over 6000 species have been studied for the bioassays. By mechanical damage to the skin, chemicals released elicit a fright reaction in conspecifics, and in many cases other species as well.

However, even for the ostariophysi, the evolutionary development of the alarm system in fish is poorly understood, hypotheses concerning the evolution of alarm signals remain as the few empirical tests done by Smith et al. (1995). They used laboratory and field experiments to demonstrate that the alarm substance of fathead minnows (Cyprinidae) is attractive to two different predators, northern pike and predaceous dividing beetles. They proposed the suggestion that the damaged skin releases alarm pheromones. For instance, the alarm pheromones of ostariophysan fish are analogous to the distress calls of some birds and mammals in that they are produced only after the sender has been captured and may function to attract other predators. Alarm signals that are released only following capture by predators may therefore be functionally distinct from other alarm signals and may have evolved through direct benefits to the sender (Smith et al., 1995). Their results clearly did not support the hypothesis of predator deterrence, but they suggested that predators are attracted by minnow alarm substance. According to the predator-attraction hypothesis, alarm pheromone functions to attract additional predators that interfere with the predation event, which allows an opportunity for the prey to escape (Smith et al., 1995). However, there is only insufficient evidence of the evolution of chemical alarm signals in fish.

There is continued laboratory work on the proximate details of the ostariophysan alarm substance system, effectiveness of fright reaction on the skin extract and the evolution of chemical alarm signals in fish.
Recently, alarm substance cells (specialised club cells) of most representatives of the ostariophysan fish were identified by Pfeiffer (1960, 1977). Since 1960 these studies are still continued in detailed progress. Unfortunately, more detailed studies of the cells producing alarm substances of giant danio *Danio malabaricus* are not available as yet (see chapter 1.3.1).

None of the natural pterin pheromones involved in fish, especially in danios, is completely identified up to now, although some suggestions are described (Hüttel et al., 1943; Pfeiffer and Reutter, 1973; Pfeiffer and Lemke, 1973). This study clearly shows that the alarm substance, 7-hydroxybiopterin from giant danio, *Danio malabaricus*, with the basic structure of pteridine was chemically identified as the first alarm pheromone among all fish studied so far.
It can be concluded that isoxanthopterin related compounds may also modify the behavioural responses amoung Ostariophysi. By using the identified component of danios, 7-hydroxybiopterin, it might be possible to demonstrate a fright reaction relationship of the alarm response in other species. In this condition, 7-hydroxybiopterin is not only the most important bioactive component in danio, but it can also act as the alarm signalling in other ostariophysan and non-ostariophysan fish.

5 Summary

Within the scope of this work, the chemical structural features and biological significance of the alarm pheromone of giant danio *Danio malabaricus* were investigated. When damaging the skin of the danio fish, club cells in the epidermis containing an alarm pheromone are broken. From these sources, pheromone is released into the environment.
The pheromone substance, which is extracted from the skin of danio fish, is responsible for the biological activity of the same species. The isolation and identification of the pheromone substance was carried out by deploying the following methods:

- Extraction of skin of the fish;
- Centrifugation and ultrafiltration of skin extracts;
- RP 18-HPLC separation of filtrate and fractionating;
- GFC (Toyoperal HW40-S gel) separation of active fraction from 3. HPLC step and fractionating;
- UV-Visible spectroscopy and TLC analysis;
- ^{1}H, ^{13}C, 2-D (^{1}H-^{13}C-COSY) NMR spectroscopy;
- LDIMS spectrometry
- Comparing the investigated data of active substance with those of proposed synthetic compounds.

An amount of 3 mg isolated natural pheromone was obtained to identify its structure by using instrumental analytical methods. Consequently the natural alarm pheromone, 7-hydroxybiopterin is elucidated. Pfeiffer´s results (1975) do not agree at all with the hypothesis of Pfeiffer and Lemke (1973) and Pfeiffer and Reutter (1973) that the alarm substance from Ostariophysi was 7-hydroxybiopterin or another fluorophore from the skin. Studying the chemistry of the alarm substance from *Phoxinus*, confirmed the results of Argentini

(1976) and the conclusion of Pfeiffer (1975) that the alarm substance is not a pterin. However, there is no doubt that 7-hydroxybiopterin is the alarm substance of danio. The lowest bioactive amount, that induced a fright reaction in bioassays carried out as described in chapter 3.2.5, was 5 mL of an aqueous solution of 7-hydroxybiopterin (0.4 µM) in 20 L test-aquarium. For specificity and selectivity of the pheromone, the bioassays were investigated with several substances, which are structure related with pterins and purines. The results presented here proved that 7-hydroybiopterin was a high specific pheromone structure. Little changes of chemical structure of this 7-hydroxybiopterin led to lose of pheromonal activity. However, in these experiments, isoxanthopterin, 6-carboxyisoxanthopterin and 6-(α-hydroxyethyl) isoxanthopterin at high amount showed the pheromonal activities too.

5 Zusammenfassung

Im Rahmen dieser Arbeit wurden die chemischen und strukturellen Merkmale sowie die biologische Bedeutung des Schreckstoffs des Danio-Fisches *Malabarbärbling* untersucht. Wenn man die Haut des Danio-Fisches verletzt, werden sogenannte „Kolbenzellen“ in der Epidermis, die ein Alarmpheromon enthält, zerstört. Dadurch wird der Schreckstoff an das Wasser abgegeben.
Die Isolierung und Identifizierung der Pheromon-Substanz, die aus der Haut des Danio-Fisches gewonnen werden konnte, wurde mit Hilfe der folgenden Methoden durchgeführt:

- Extraktion der Fischhaut;
- Zentrifugation und Ultrafiltration von Hautextrakten;
- RP-18-HPLC;
- GFC-(Toyoperal HW40-S Gel)-Trennung;
- UV-VIS Spektroskopie und UV-VIS TLC-Analyse;
- ^{1}H, ^{13}C, 2-D-NMR-Spektroskopie (^{1}H-, ^{13}C-COSY);
- LDIMS-Spektrometrie

Abschließend erfolgte der Vergleich der untersuchten Daten des Schreckstoffs mit denen der synthetischen Komponenten.

3 mg des isolierten natürlichen Pheromons wurden verwendet, um dessen Struktur mit instrumentellen analytischen Methoden zu identifizieren. Das natürliche Alarmpheromon wurde als 7-Hydroxybiopterin identifiziert. Ergebnisse von Pfeiffer (1975) stimmen nicht mit der Hypothese von Pfeiffer und Lemke (1973) und Pfeiffer sowie Reutter (1973) überein, daß der Schreckstoff 7-Hydroxybiopterin oder andere Fluorphore aus der Haut von Ostariophysi sein könnte. Die natürlichen chemischen Eigenschaften des Schreckstoffes von *Phoxinus* bestätigen die Ergebinisse von Argentini (1976) und von Pfeiffer (1975), daß es sich bei diesem isolierten Schreckstoff nicht um Schreckstoff 7-

Hydroxybiopterin handelte. Es besteht allerdings kein Zweifel darin, daß 7-Hydroxybiopterin den Schreckstoff von Danio repräsentiert. Bei Biotests mit 5 mL einer 0.4 µM 7-Hydroxybiopterin-Lösung in einem 20 L Aquarien konnte eine Schreckreaktion ausgelöst werden. Um die Spezifität und Selektivität der Pheromone zu verifizieren, wurde der Biotest mit verschiedenen Substanzen aus der Verbindungsklasse der Pterine und Purine durchgeführt. Wie die Ergebnisse der Biotests zeigten, handelt es sich bei 7-Hydroybiopterin um einen Schreckstoff mit einer hohen spezifischen pheromonstruktur. Kleine Änderungen der Struktur führten zum Verlust der pheromonalen Aktivität. Bei großen Menge zeigten jedoch auch Isoxanthopterin, 6-Carboxyisoxanthopterin und 6-(α-Hydroxyethyl)-isoxanthopterin eine Schreckreaktion induzierende Aktivität.

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Appendix A. UV spectra of purine and pterin derivatives

Appendix A1

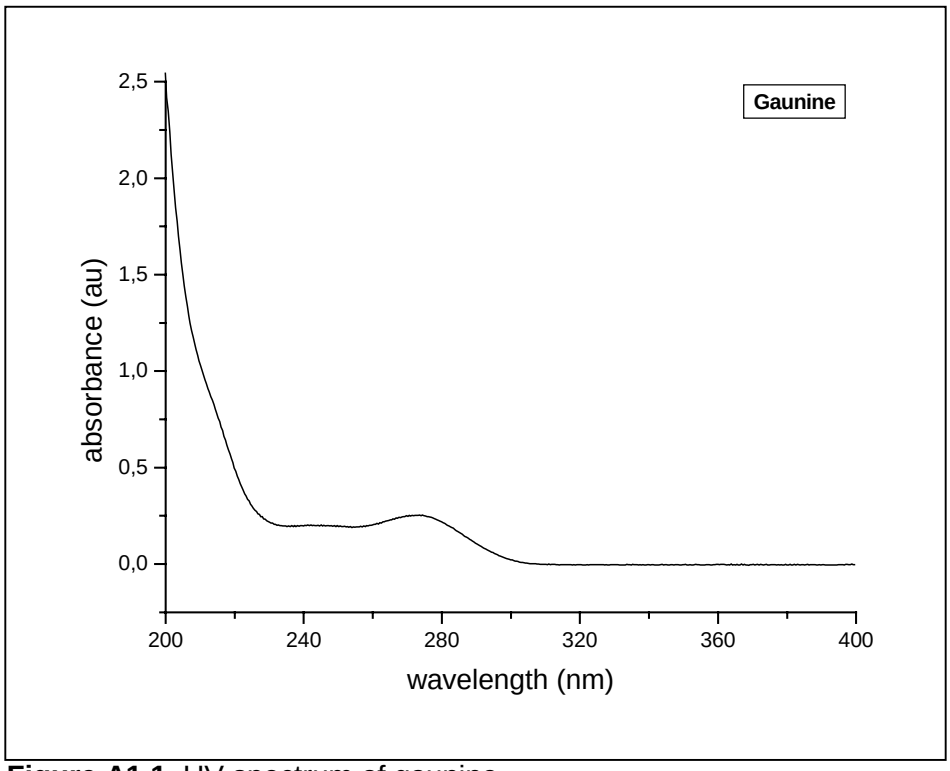

Figure A1.1. UV spectrum of gaunine

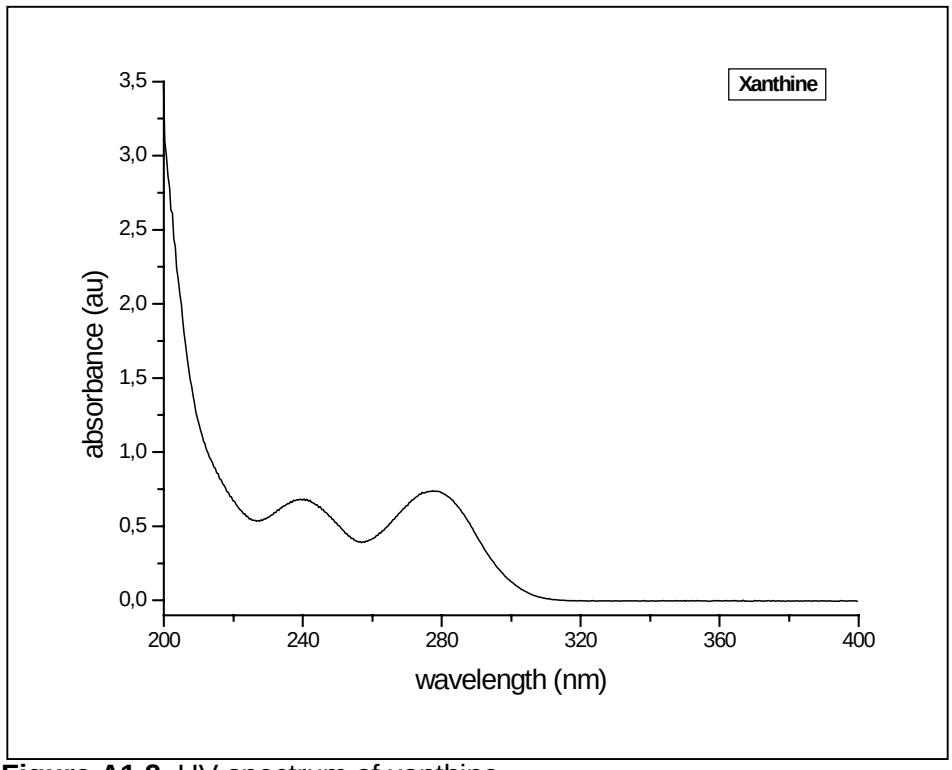

Figure A1.2. UV spectrum of xanthine

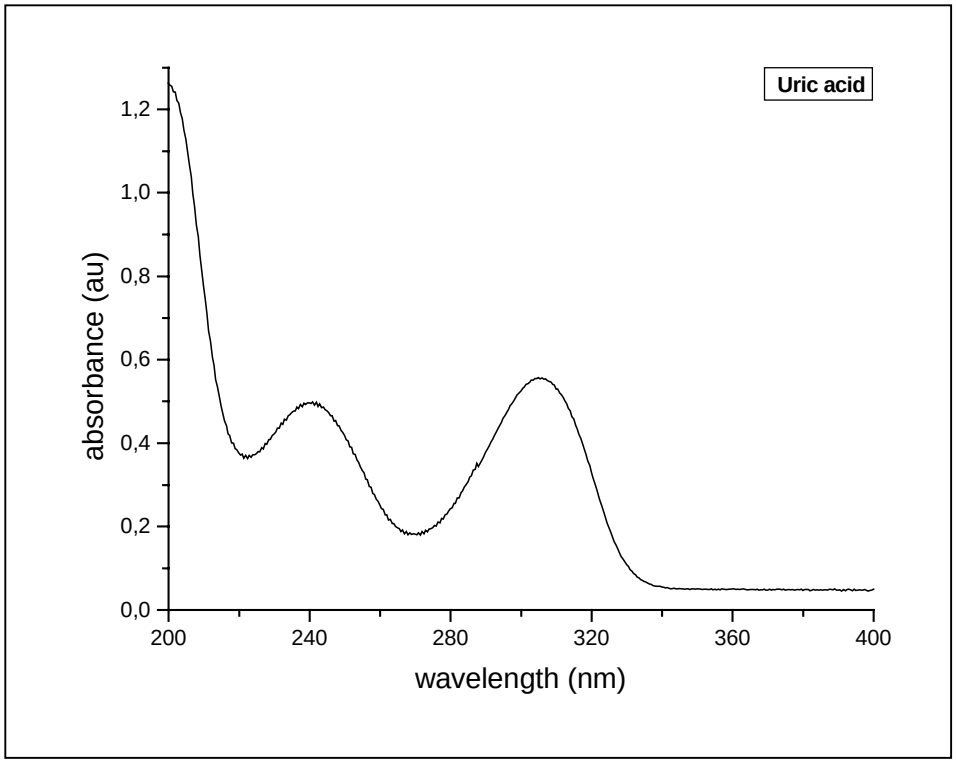

Figure A1.3. UV spectrum of uric acid

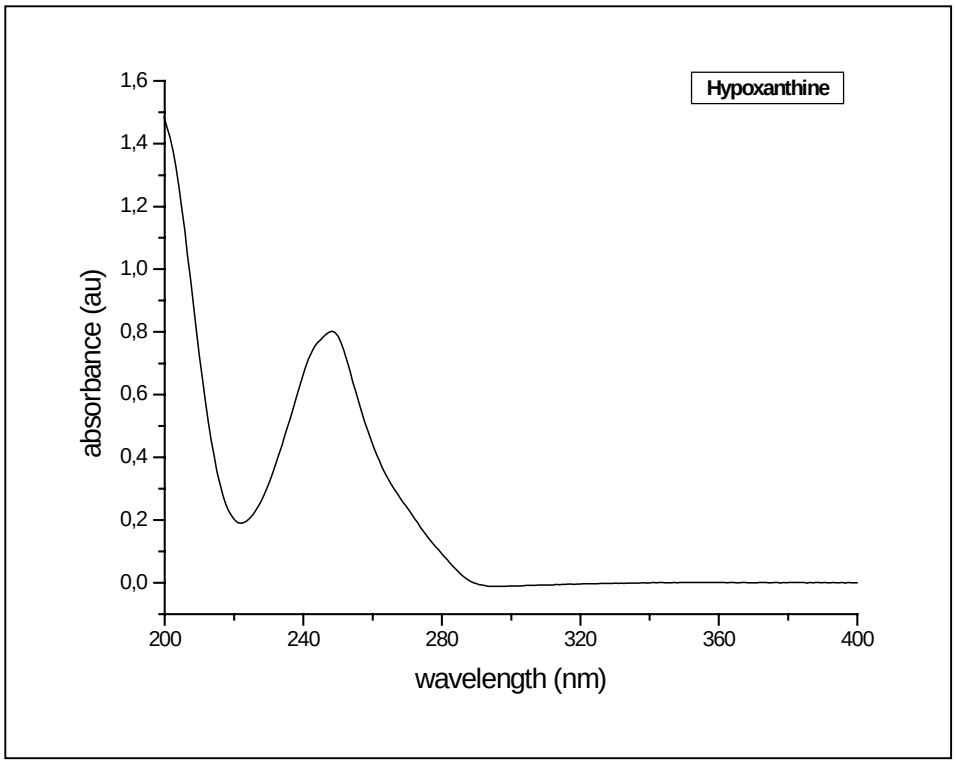

Figure A1.4. UV spectrum of hypoxanthine

Appendix A2

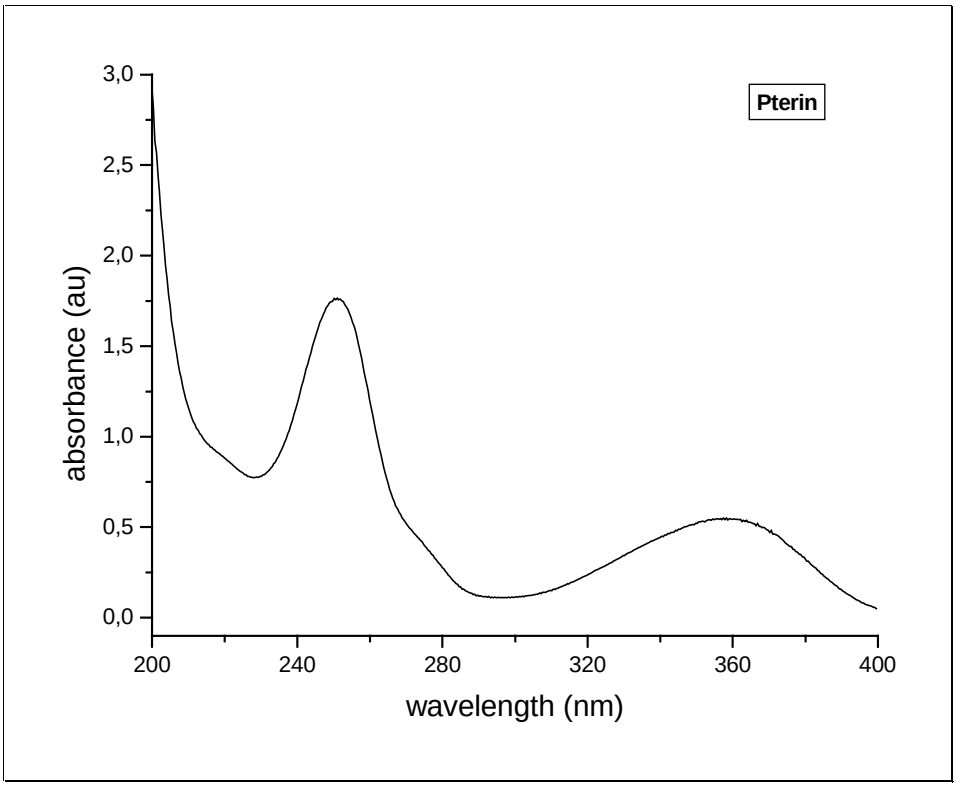

Figure A2.1. UV spectrum of pterin

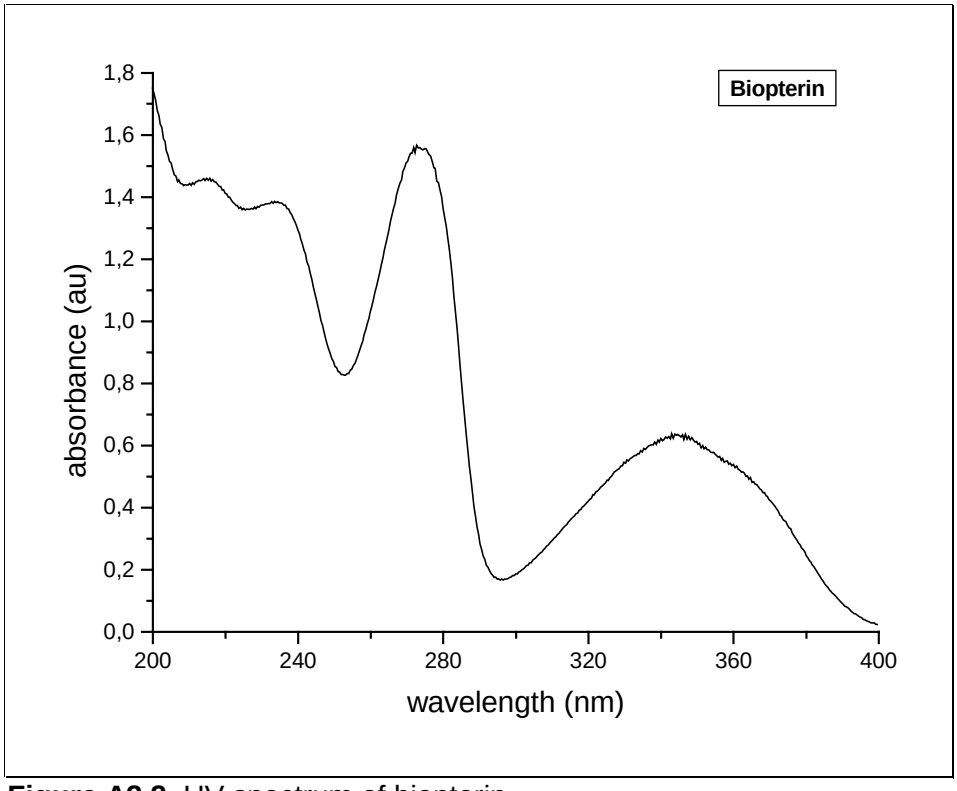

Figure A2.2. UV spectrum of biopterin

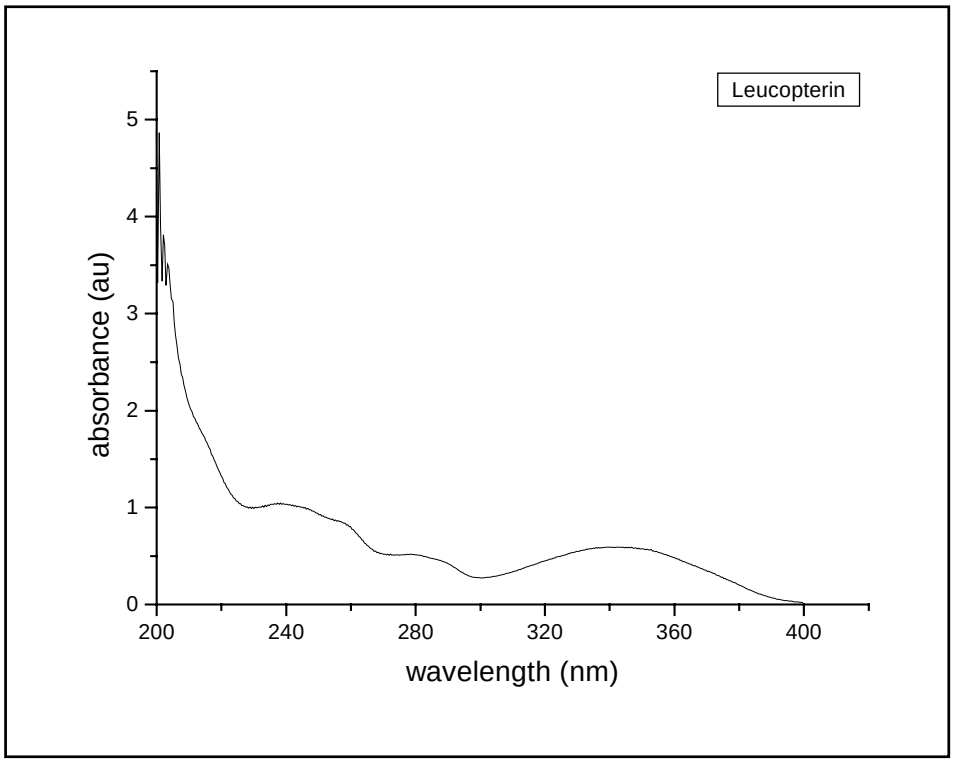

Figure A2.3. UV spectrum of leucopterin

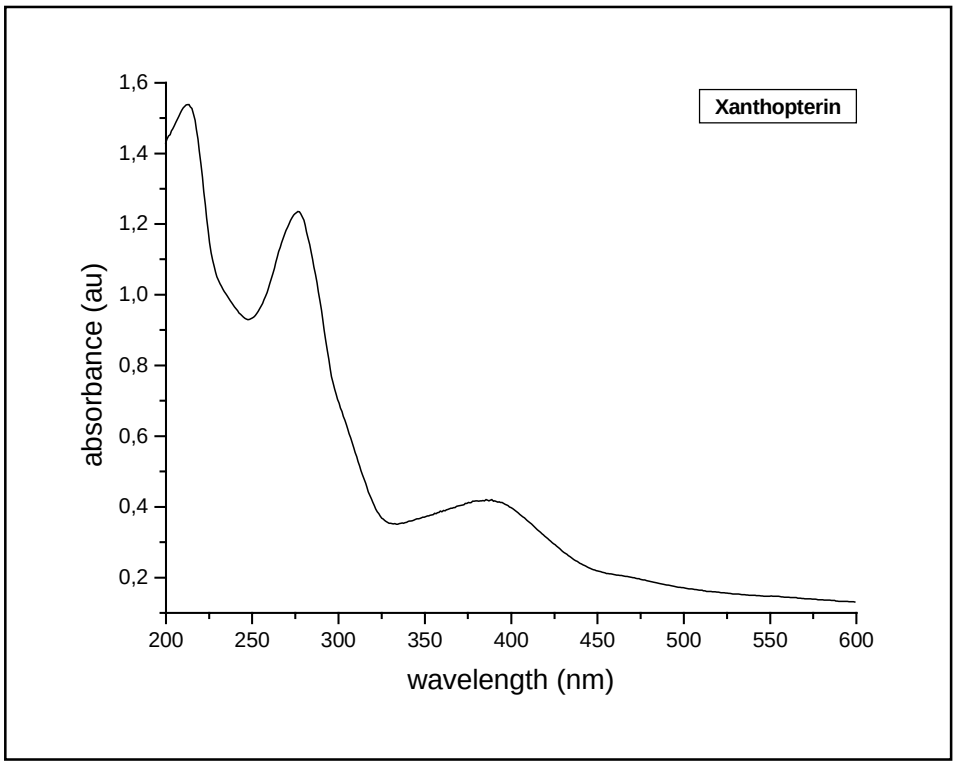

Figure A2.4. UV spectrum of xanthopterin

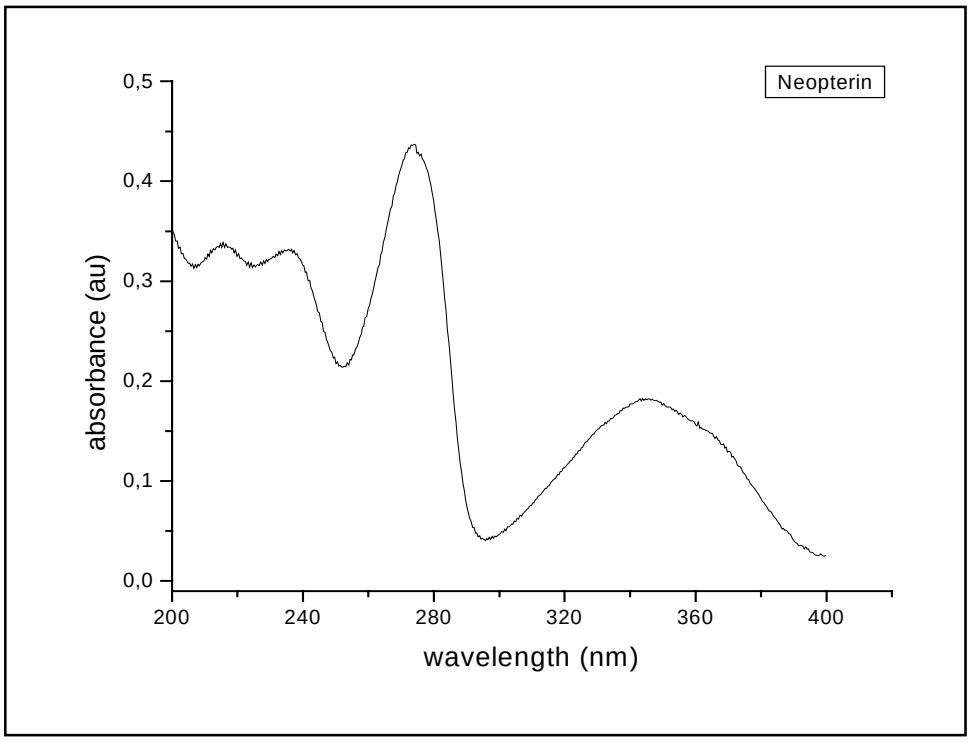

Figure A2.5. UV spectrum of neopterin

Appendix A3

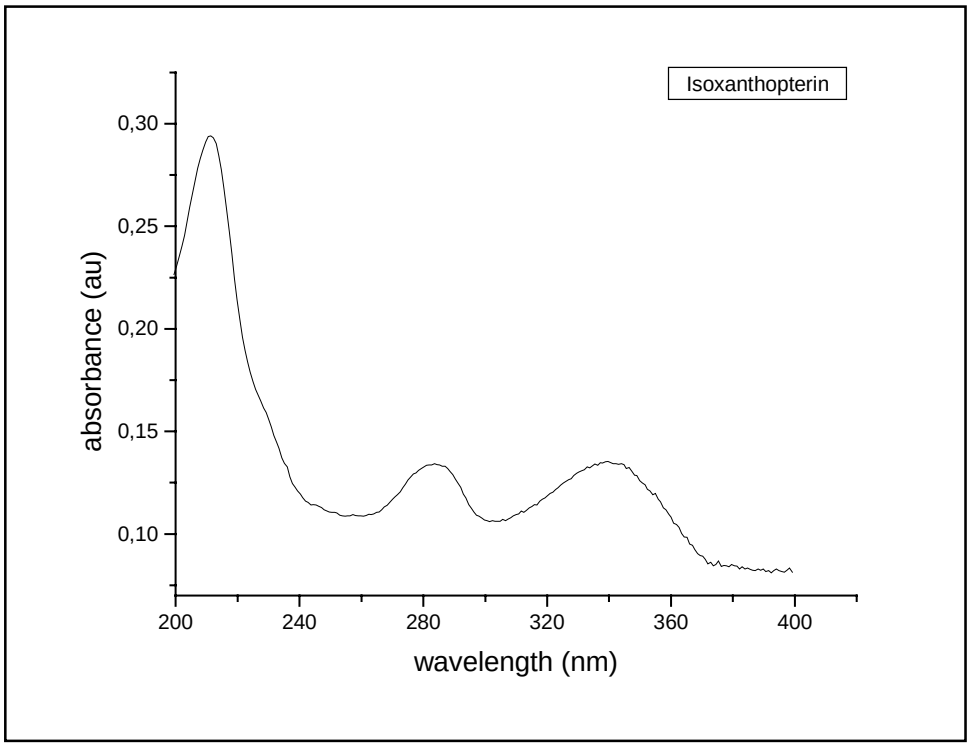

Figure A3. UV spectrum of isoxanthopterin

Appendix A4

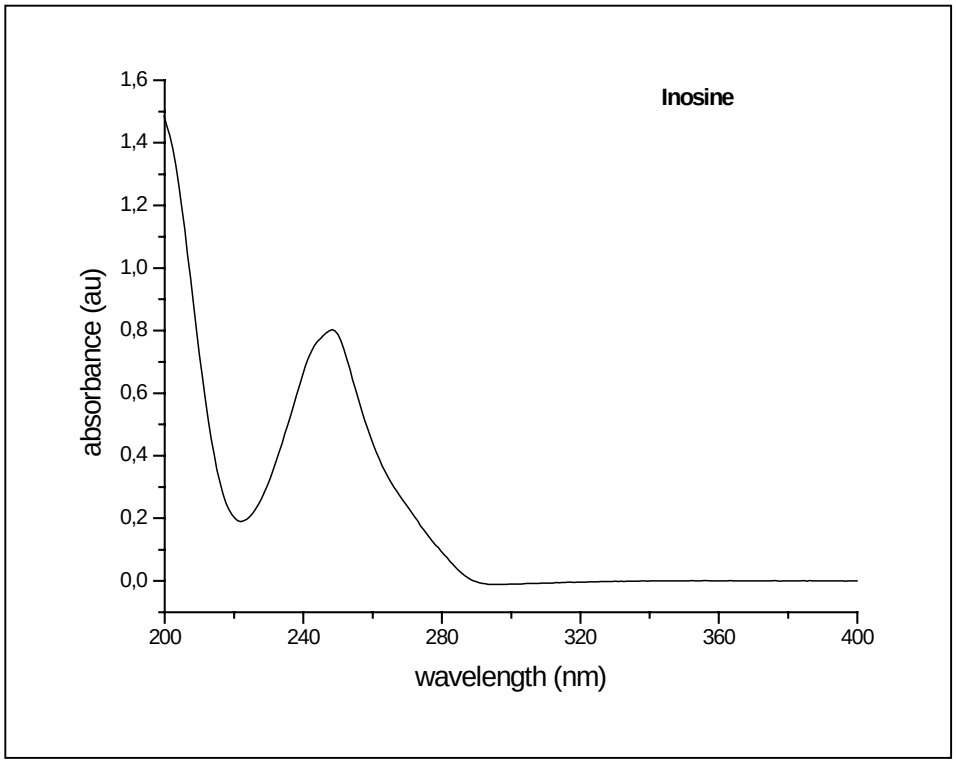

Figure A4.1. UV spectrum of inosine

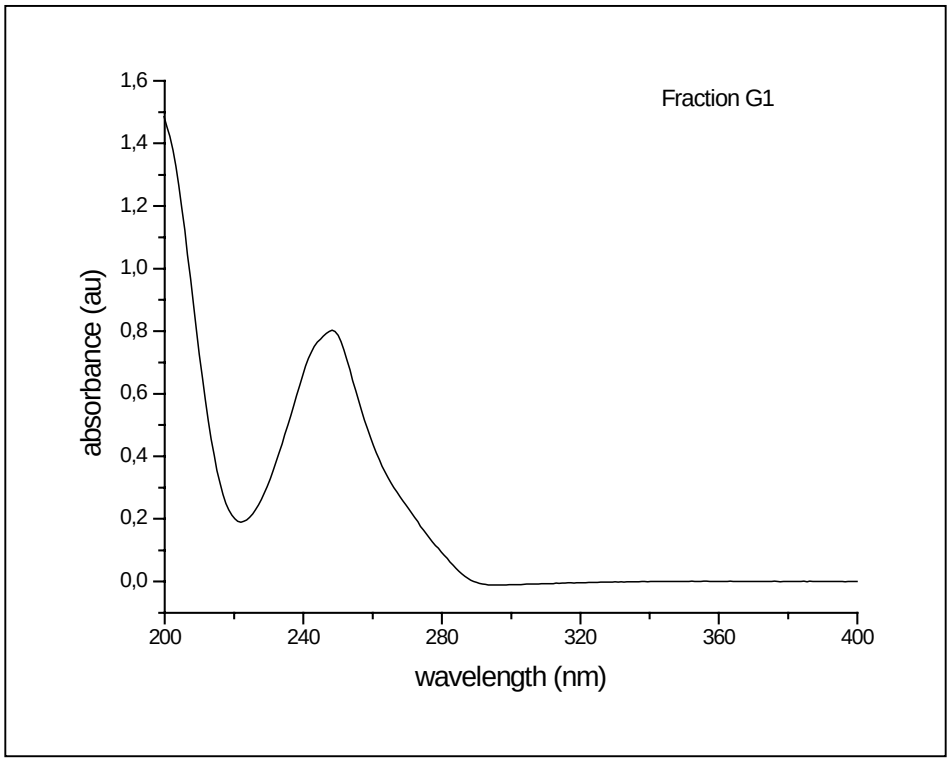

Figure A4.2. UV spectrum of fraction G1

Appendix B. NMR spectra of standard 1 and standard 2

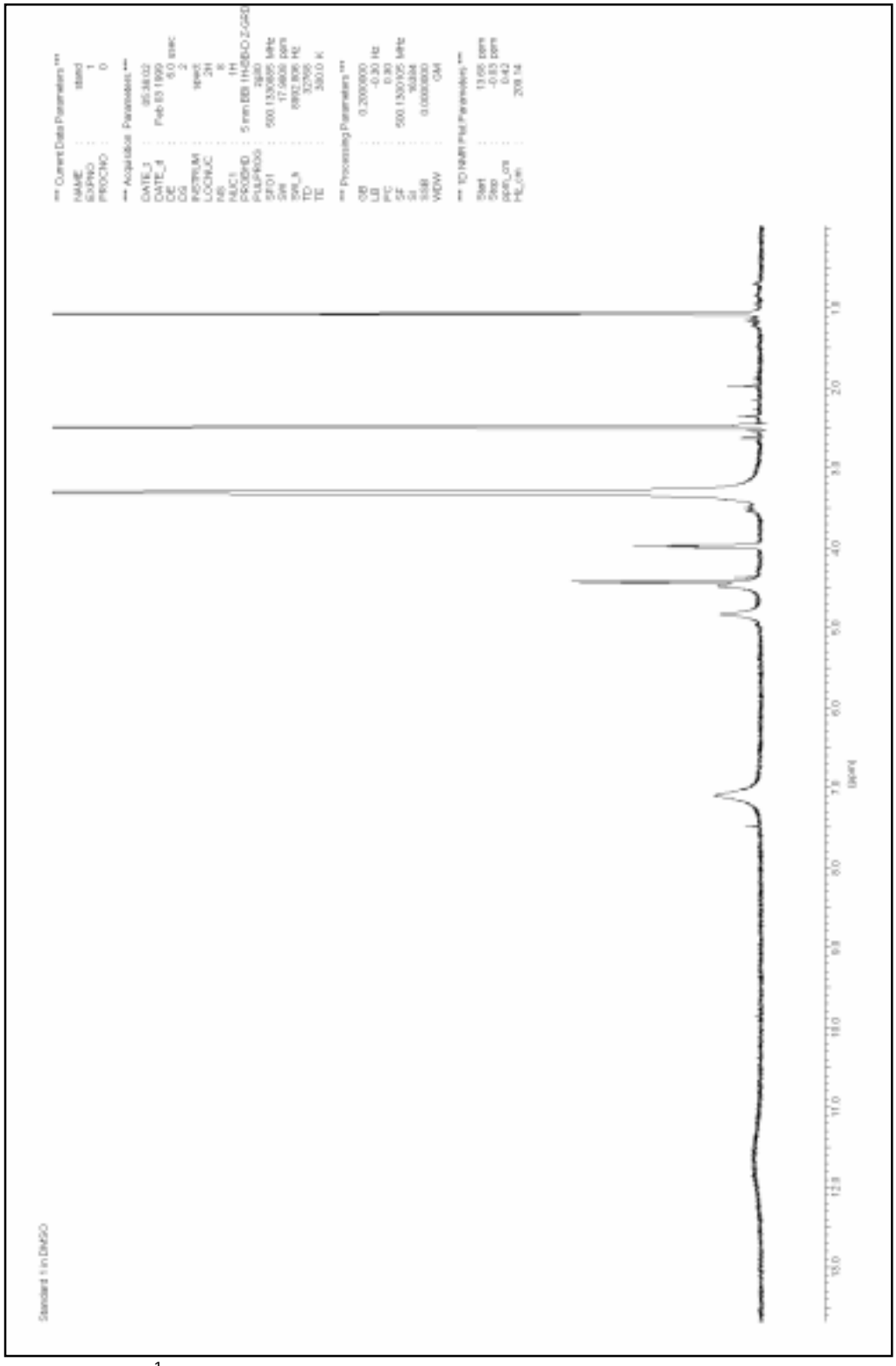

Figure B1.1. ^{1}H NMR of standard 1

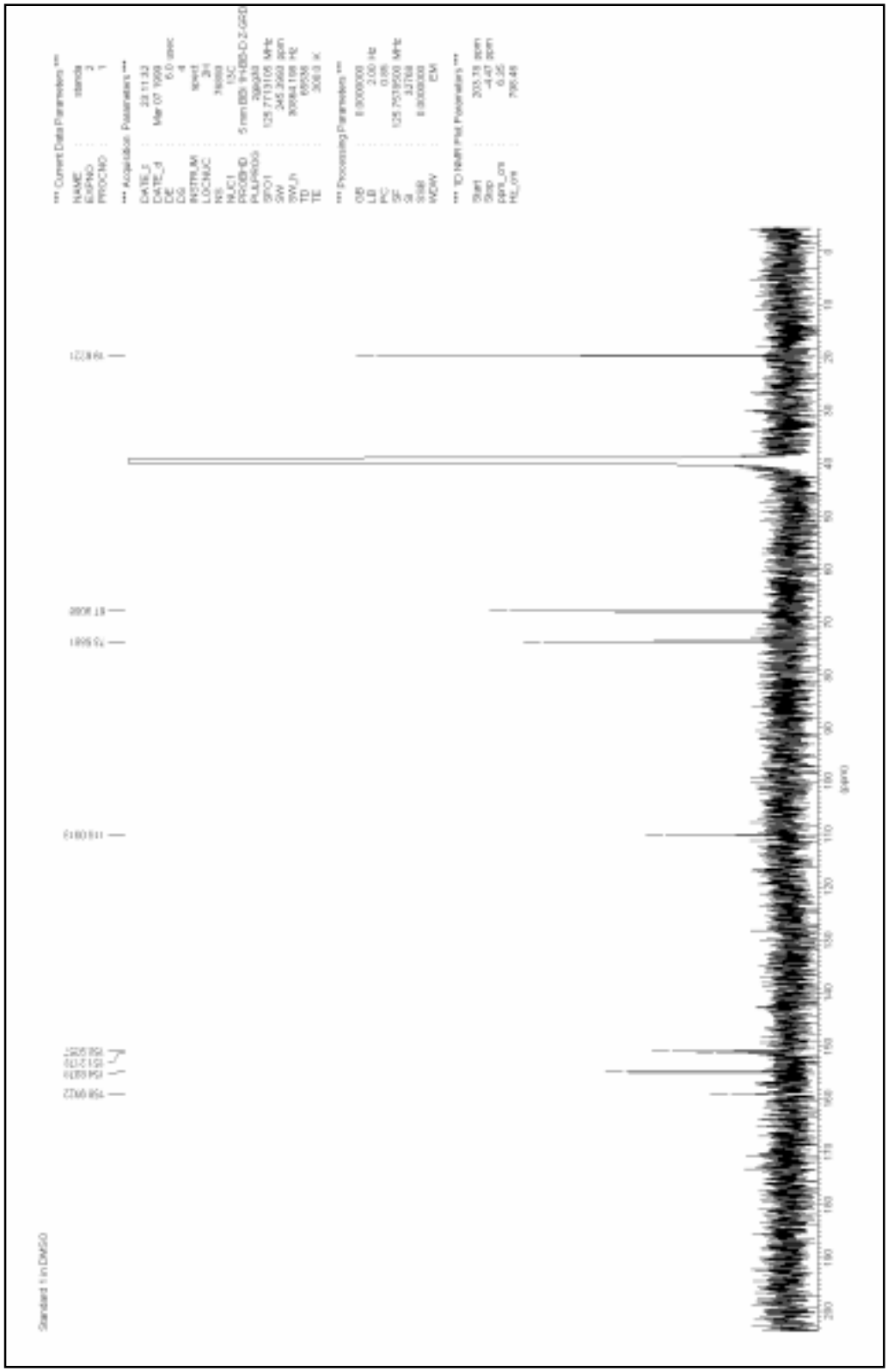

Figure B1.2. ^{13}C NMR of standard 1

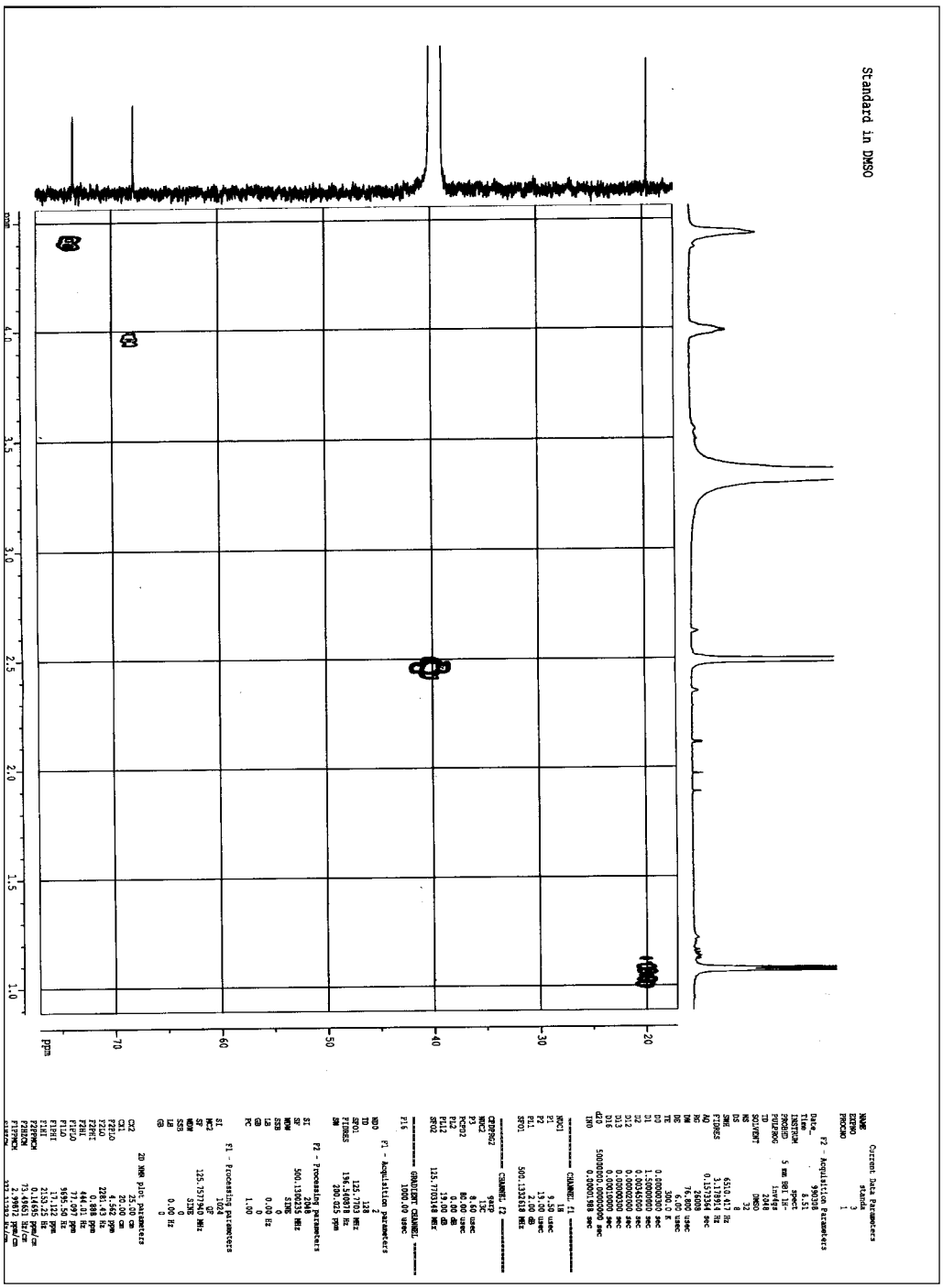

Figure B1.3. ^{1}H-^{13}C-COSY of standard 1

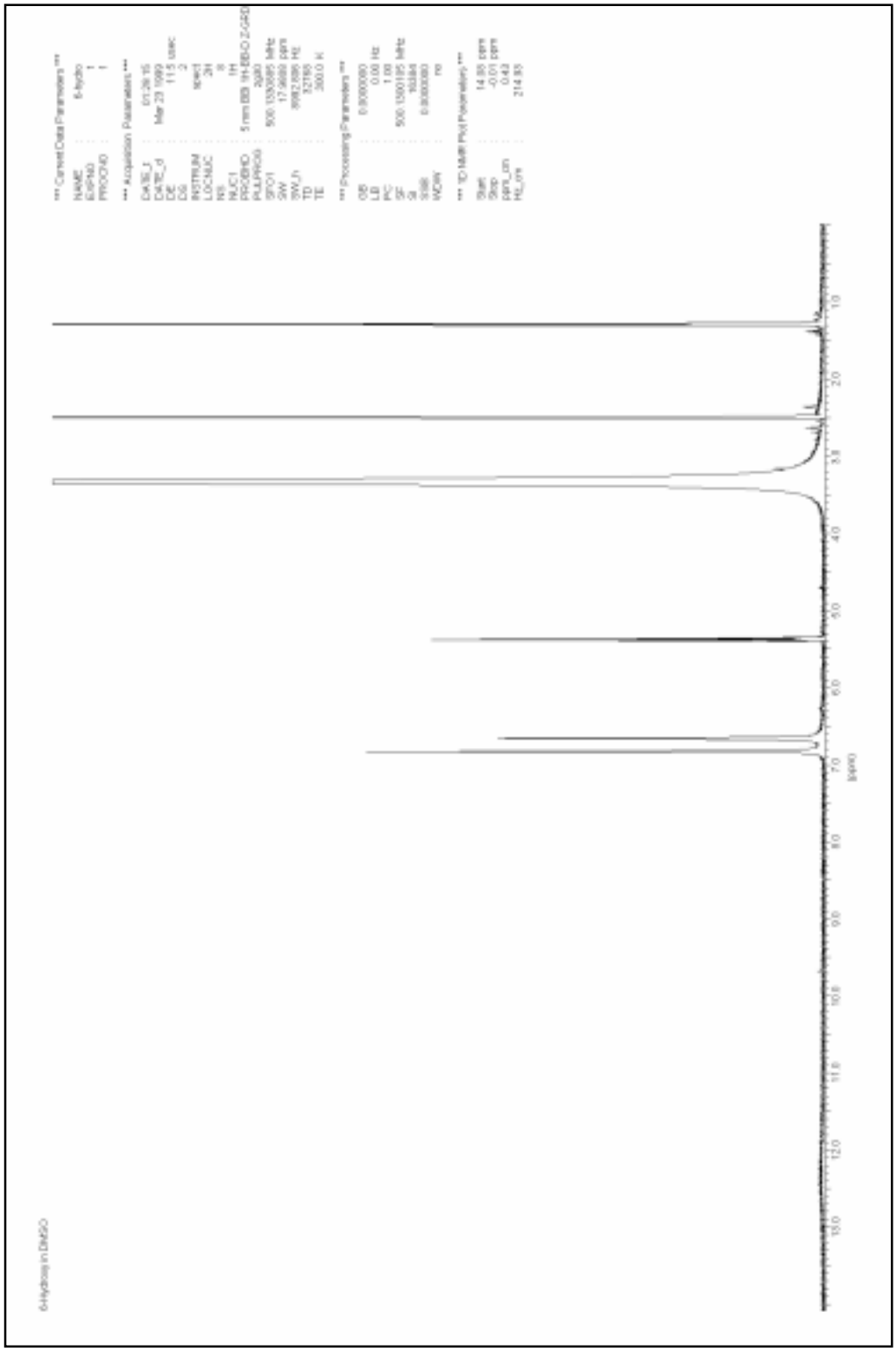

Figure B2.1. ^{1}H NMR of standard 2

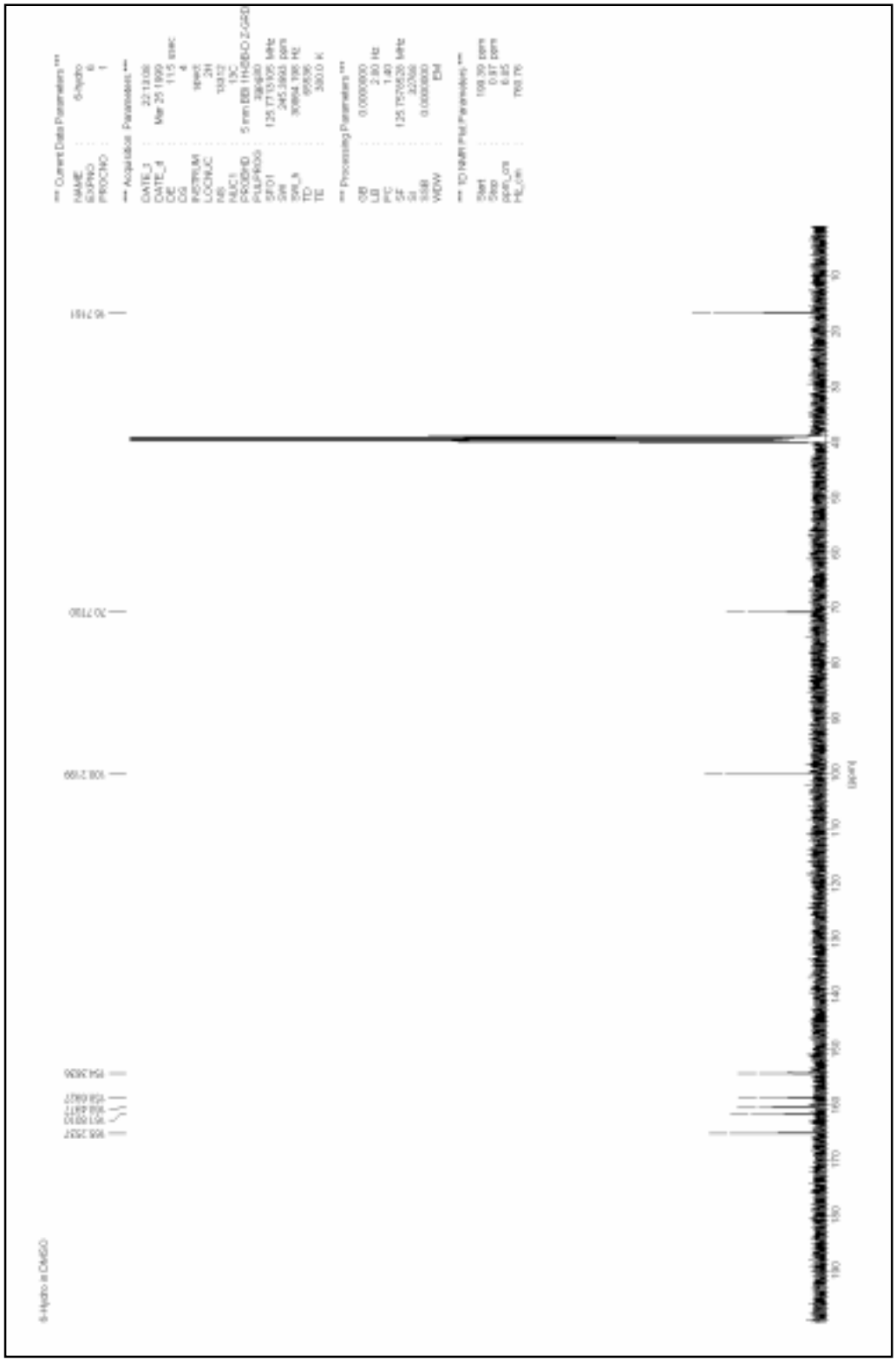

Figure B2.2. ^{13}C NMR of standard 2

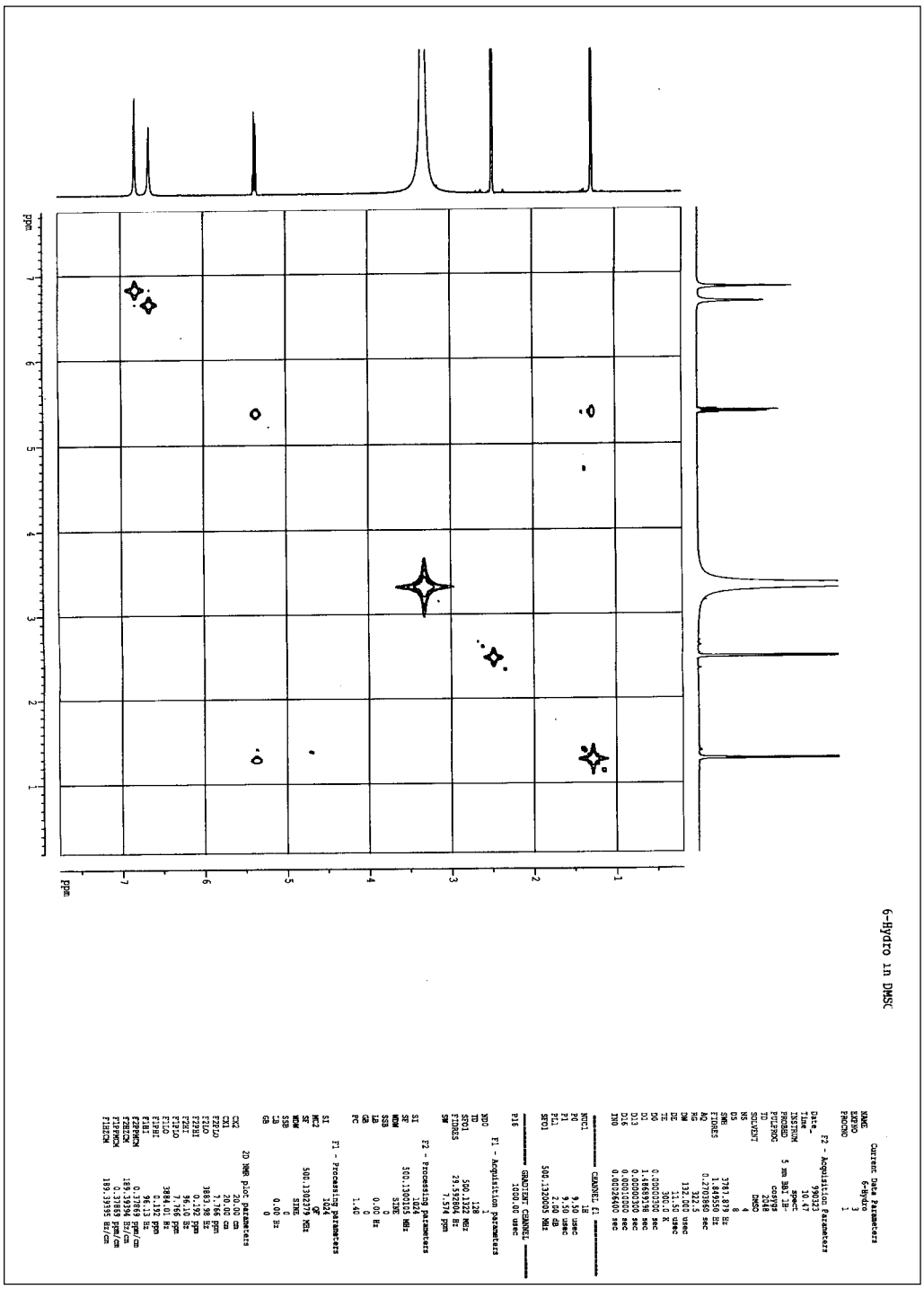

Figure B2.3. ^{1}H-^{13}C-COSY of standard 2

Appendix C. Mass spectra of standard 1 and standard 2

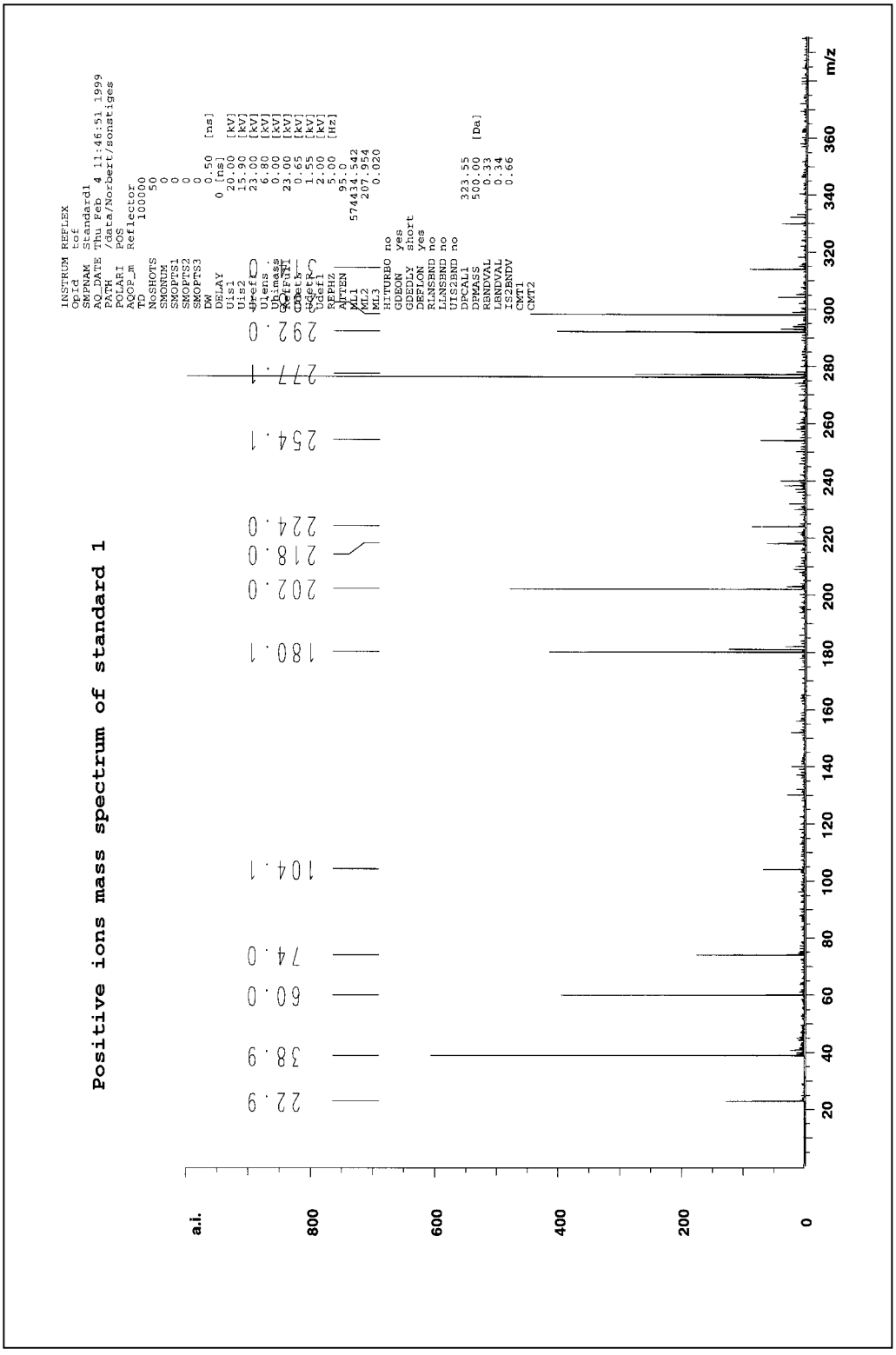

Figure C1.1. Positive ions mass spectrum of standard 1

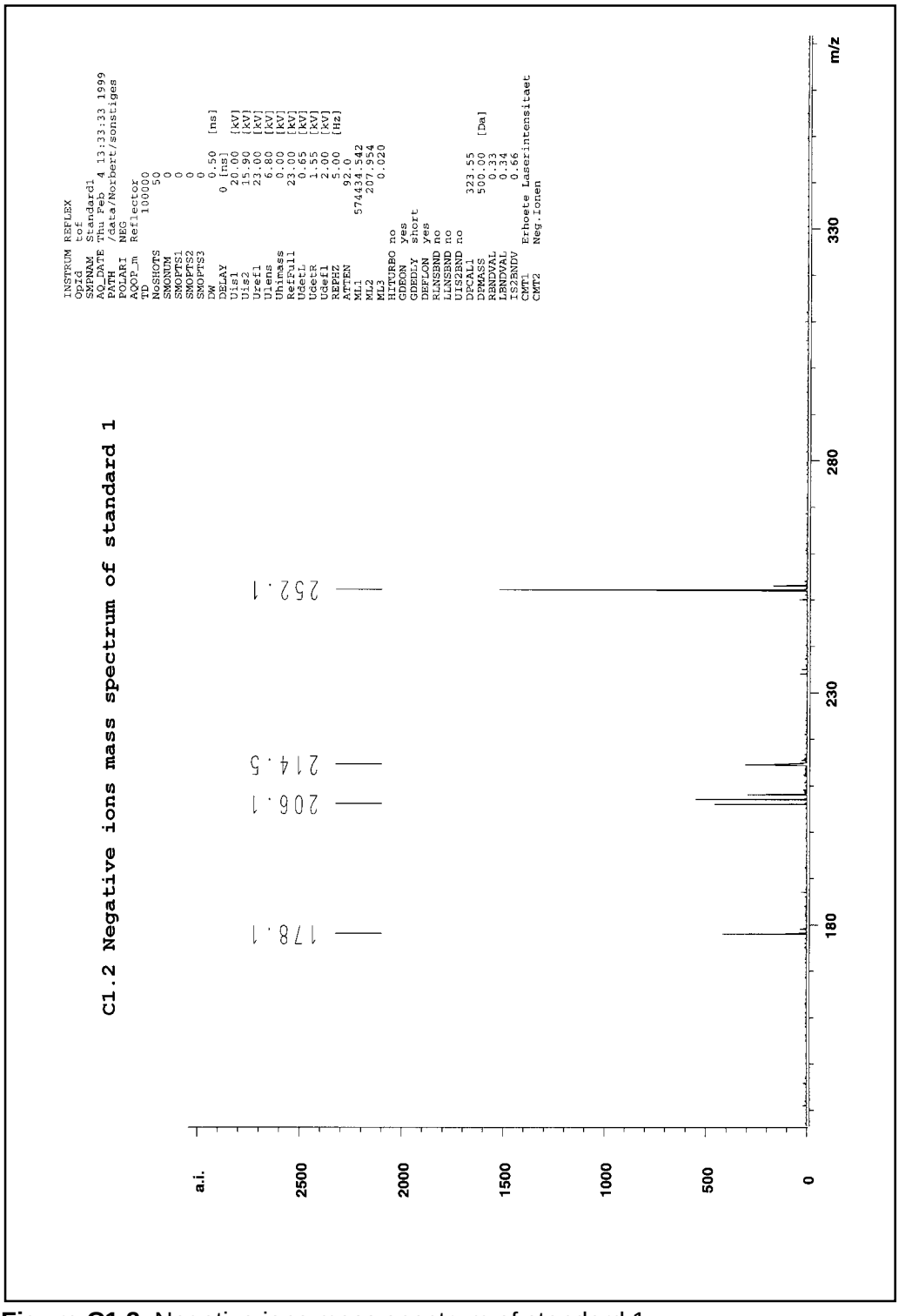

Figure C1.2. Negative ions mass spectrum of standard 1

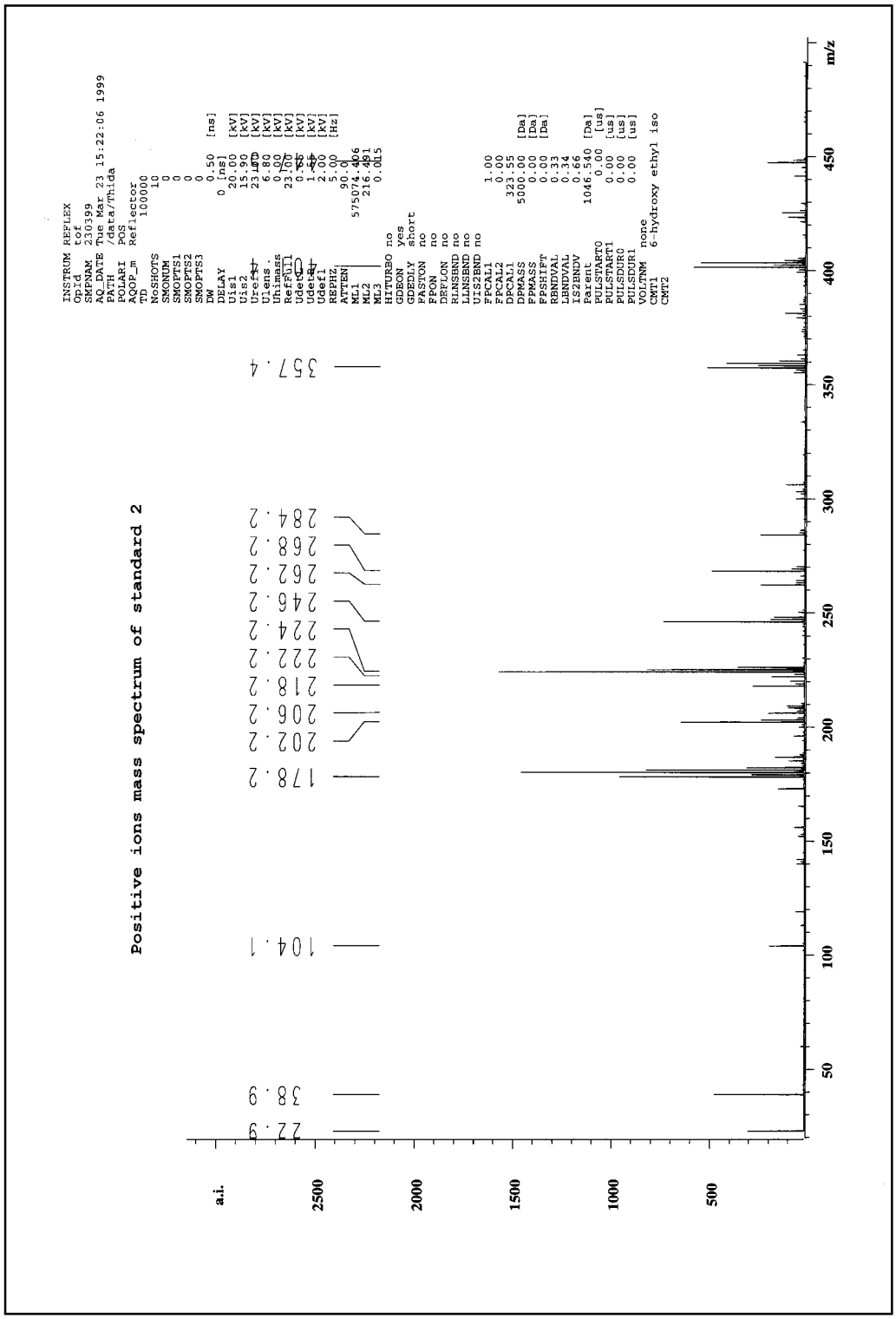

Figure C2.1. Positive ions mass spectrum of standard 2

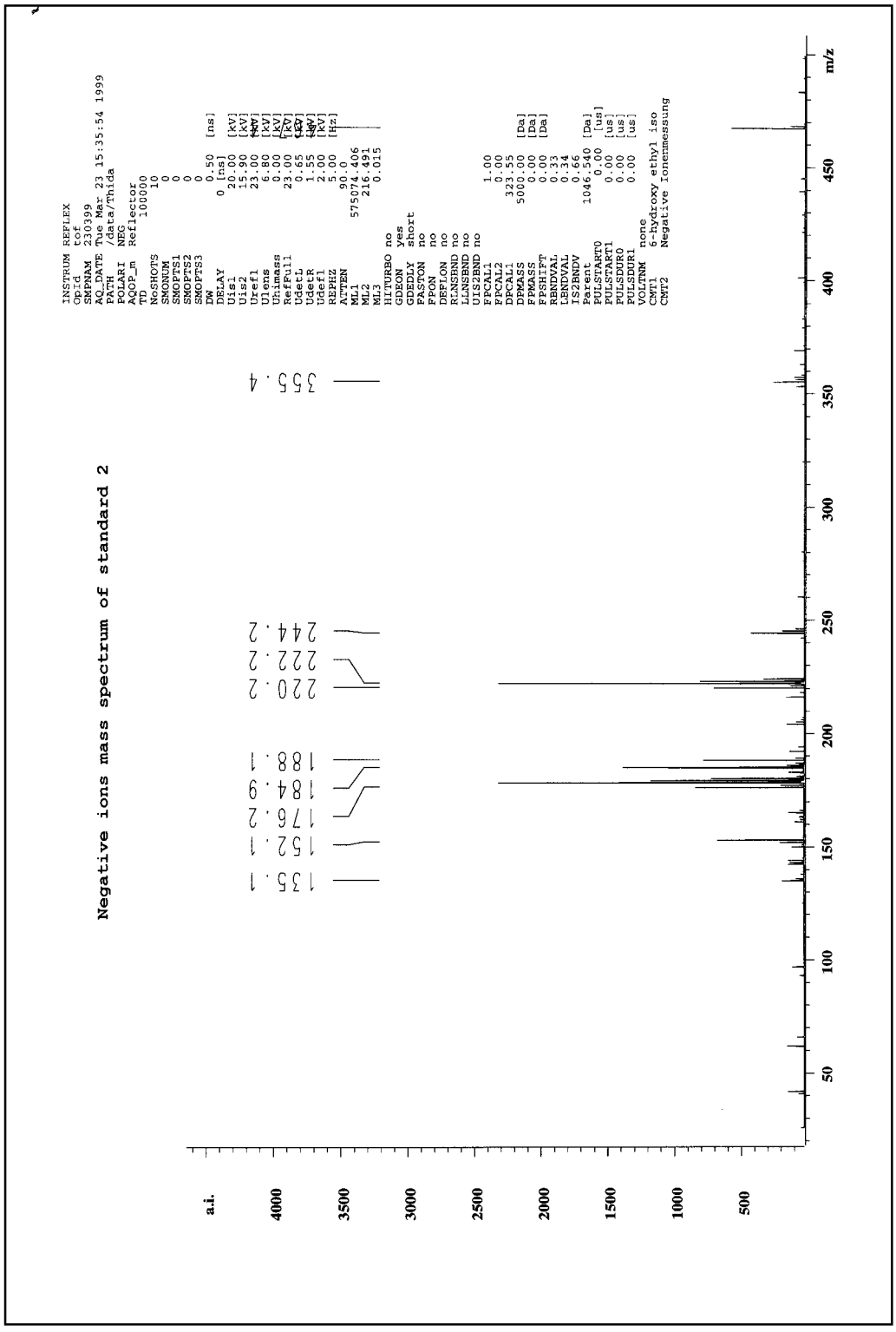

Figure C2.2. Negative ions mass spectrum of standard 2

Appendix D. NMR spectra of fraction 3.1.1.1 and inosine

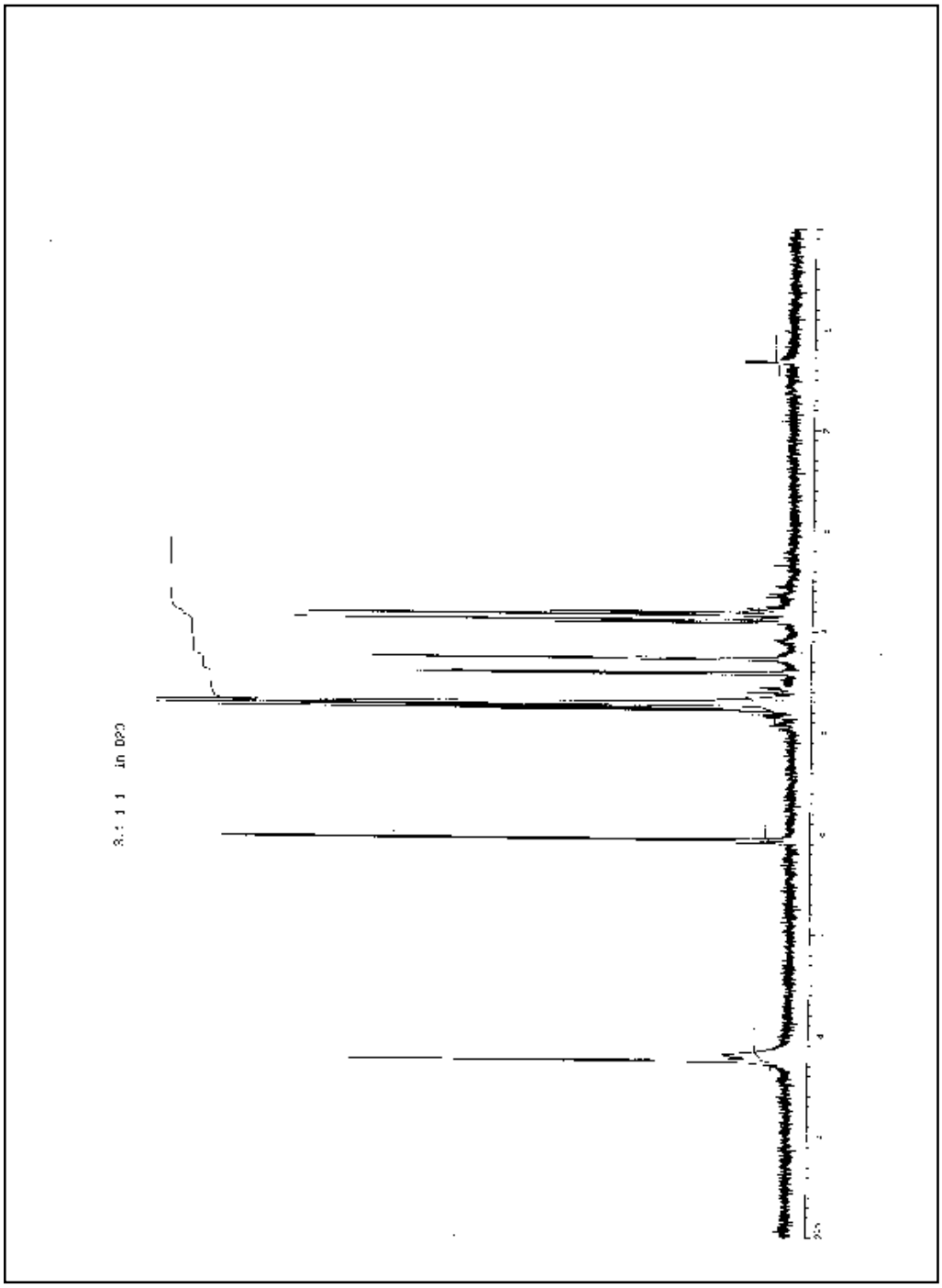

Figure D1.1. ^{1}H NMR of fraction 3.1.1.1

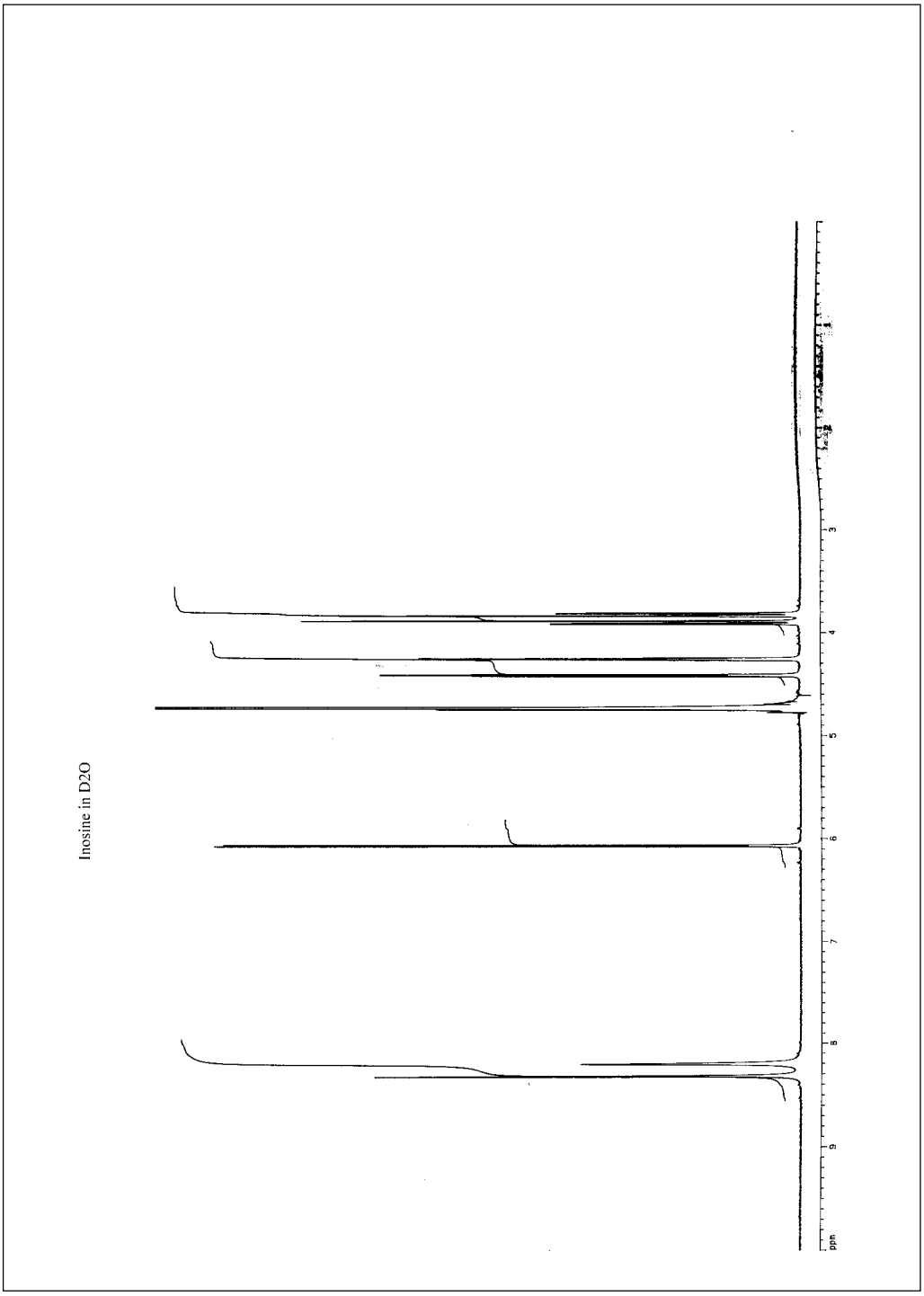

Figure D1.2. ^{1}H NMR of inosine

Abbreviations

°C	Grad celsius
cm	Centimetre
COSY	Correlation spectroscopy
DMSO	Dimethylsulfoxide
D_2O	Deuteriumoxide
D-	Dextro
g	Gramme
GFC	Gel filtration chromatography
h	Hour
hPa	Hectopascal
HPLC	High-performance liquid chromatography
kD	Kilodalton
L-	Levo
LC	Liquid chromatography
LDIMS	Laser desorption ionisation mass spectrometry
MALDI-TOF	Matrix-assisted laser desorption/ionisation-time of flight
mg	Milligramme
min	Minute
mL	Millilitre
mm	Millimetre
MHz	Megahertz (Frequency)
m/z	Mass/charge
µL	Microlitre
µm	Micrometre
µM	Micromole per litre
NAD	Nicotinamide-adenine dinucleotide
NADH	Reduced NAD
NMR	Nuclear magnetic resonance
nm	Nanometre
ng	Nanogramme
p.a.	Pro analysis
Pa	Pascal
PDA	Photodiode array detector
ppm	Parts per million
Psi	Psicose

RP	Reversed-phase
rpm	Resolutions per minute
sec	Second
TLC	Thin layer chromatography
T_R	Retention time
UV	Ultraviolet
v/v	Volume/volume
Vis	Visible
XD	Xanthine dehydrogenase
XO	Xanthine oxidase
λ_{max}	Wavelength in nm

Curriculum Vitae

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1997 September	Enrol as a Ph.D. candidate in Chemistry Department, University of Oldenburg, Germany

Erklärung:

Hiermit erkläre ich, daß ich die vorliegende Dissertation selbständig angefertigt und nur die angegebenen Hilfsmittel verwendet habe.

Oldenburg d. 21.03.2000

Thida Win