Structure-Activity Relationships in Glucosinolates as Oviposition Stimulants of the Cabbage Root Fly, *Delia radicum* (L.)

By

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ABSTRACT

STRUCTURE-ACTIVITY RELATIONSHIPS IN GLUCOSINOLATES AS OVIPOSITION STIMULANTS OF THE CABBAGE ROOT FLY, *DELIA RADICUM* (L.)

Neil Patrick Chilcott

A statistically sound and quantifiable bioassay procedure was developed for determining the response of adult *Delia radicum* to oviposition stimulants. The method was based on the use of surrogate leaves coated with test compounds.

All of the test compounds were of purity 99% or greater. Twelve of the tested compounds were synthesised the remaining twenty four were either donated or available commercially. Attempts to follow literature methods to synthesise glucosinolates were unsatisfactory and an investigation of the procedures led to a number of synthetic modifications. Despite various attempts, the failure to sulphate a thiohydroximate to produce a glucosinolate was not resolved.

Attempts to correlate oviposition stimulus with chemical structure produced a very significant finding. The results showed that a wide variety of chemically dissimilar compounds were effective stimulants providing they contained an S=O group. Thus sulphoxides, sulphones, sulphinic, sulphonic acids and their derivatives were all effective. Thiols and thioethers were non-stimulant, as were naturally occurring glucosinolates which had been chemically modified by the removal of the oxime sulphate group.

The relative effectiveness of the oviposition stimulants was examined by determining the number of eggs laid on surrogate leaves relative to a prop-2-enylglucosinolate (sinigrin) standard over a range or concentrations. Statistical modelling of the data collected

produced a maximum relative number of eggs laid (Y_{MAX}) at an optimum concentration (C) for each compound.

It was not found possible to produce a single parameter combining Y_{MAX} and C, neither did any other structure-activity feature emerge from the study.

CONTENTS

TABLES

FIGURES

PLATES

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AUTHOR'S DECLARATION

At no time during the registration for the degree of Doctor of Philosophy has the author been registered for any other University award.

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Signed: N. P. Chilcott

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1. INTRODUCTION

1.1 CHEMICAL ECOLOGY

As defined by the International Society of Chemical Ecology, chemical ecology is "the study of structure, function, and biosynthesis of natural products; their origin, and their importance at all levels of ecological organisation; their evolutionary origin, and their application to social needs". Within the kingdom of flowering plants alone it is estimated that over 100,000 chemicals are produced through the growth and development of more than 2,000,000 species (Metcalf and Metcalf, 1992). The normal physiology of plant growth and reproduction does not utilise the majority of these secondary compounds which include over 6000 alkaloids, 3000 terpenoids, 4000 phenylpropanoids, 1000 flavanoids, 500 quinones, 650 polyacetylenes and 400 amino acids (Harborne, 1988). For example, morphine only occurs in two species of poppy and although used and abused by man it is of no known benefit to the plant. Similarly, penicillins are produced by only a few species of fungi, and man has learnt to utilise these as antibiotics, but they do not seem to have any useful purpose in the micro-organisms that produce them (Mann, 1987). It is only on careful examination of the co-evolution of plants with other organisms, in particular insects that this huge array of organic chemicals is seen to have a function.

Humans are familiar with many of the biochemicals that are produced by plants associated with their colour, odour and taste. It is only relatively recently that the role of many plant natural products has been recognised as having an adaptive advantage to the plant by acting as semiochemicals and conveying specific behavioural messages to species involved in ecological interrelations of food webs to interacting organisms. Semiochemicals are categorised further in Figure 1.

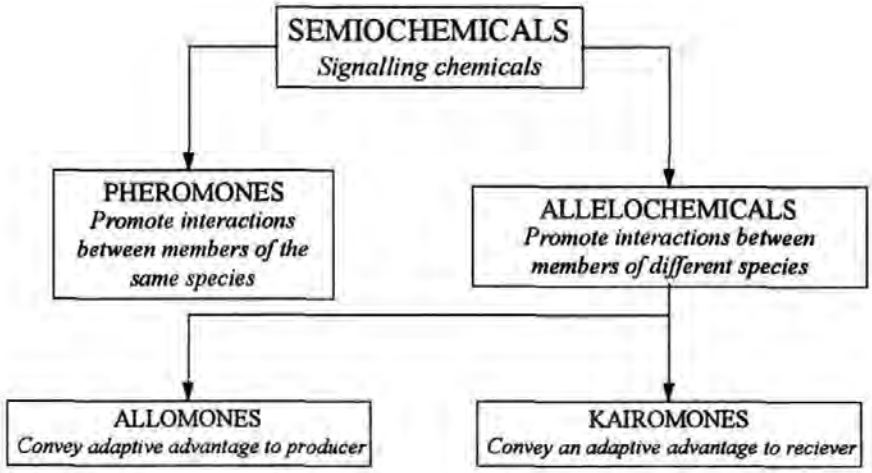


FIGURE 1 CLASSIFICATION OF SEMIOCHEMICALS

If attention is confined to animal-plant relationships there are many allelochemicals which produce olfactory or gustatory stimuli that convey specific behavioural messages to certain insects. Compounds conveying adaptive advantages to the insect receiver are referred to as Kairomones (Greek Kairos, opportunistic), and as Allomones if they convey adaptive advantage to the plant producer (Kogan, 1982). The same chemical compound may act both as an allomone by protecting the plant against attack by herbivores in general and as a kairomone by stimulating the feeding of specialist herbivores. Some kairomones may be synthesised by a wide range of plant genera or families and therefore such kairomones may regulate host plant selection by insect herbivores that are monophagous, stenophagous or oligophagous.

Host plant selection by phytophagous insects involves the sequential effects of plant produced semiochemicals on insect behaviour through host finding, feeding, oviposition, and growth and development (Kogan, 1976). In this process, plant allelochemicals influence insect behaviour by acting in a number of ways (Table 1).

ALLOMONES	
Repellents	Orientates insects away from the plant
Excitants	Initiate or accelerate movement
Suppressants	Inhibit biting or piercing
Deterrents	Deter feeding or oviposition
Antibiotics	Disrupt normal growth and development
Antixenotics	Disrupt normal host selection
KAIROMONES	
Attractants	Orientates insects toward the plant
Arrestants	Slow down or stops insect movement
Excitants	Elicits biting, piercing or oviposition

TABLE 1 SEMIOCHEMICALS INFLUENCING INSECT BEHAVIOUR

A wide range of plant semiochemicals have been identified as acting as volatile attractants for associated insect species. The attractiveness of these compounds arises from them being carried by the wind and is relatively long range. Many attractants act largely as kairomones benefiting the insect but others, including floral volatiles involved in pollination, act as synomones benefitting both the plant through pollination and the insect by rewards of nectar and pollen, or through aggregation or lek formation which lead to mating (Williams, 1983). Many of the key odours are released from certain plant tissues by highly specialised glands such as osmophores and glandular trichomes (Fahn, 1979). Air temperature is an important factor in the rates of production and release. For example orchid flowers do not produce scent on cool, overcast days or at night correlating with the behaviour of the Euglossini bee as these insects are inactive during dull weather and at night (Williams, 1981). Plants of the species *Araceae* produce heat that aids in the volatilisation of odourants such as skatole and indole (Meeuse, 1978).

A number of plant secondary compounds have been identified as non-volatile kairomones of relatively high molecular weight which act as arrestants, feeding stimulants (phagostimulants), and oviposition stimulants. The insects response to these kairomones is produced by direct contact of the substance with chemoreceptors, usually located on the insect tarsi but sometimes present on the maxillary palpi or ovipositor (Schoonhoven *et al.*, 1985; Städler and Buser, 1984).

1.2 PEST MANAGEMENT

The world population is increasing rapidly and is predicted to stabilise only once it has reached 12-15 billion people. Consequently this will lead to a similar increase in the demand for food the majority, and ultimately all of which, has to be provided by plants. As there is only a finite amount of space suitable for growing crops, agriculture has to become more intensive, especially in the Third World countries. At present, as in the majority of countries in Europe and the United States, this would require the use of fertilisers, insecticides, herbicides and fungicides. However it is becoming clear that this Western form of farming is having a detrimental affect on the environment. Problems that have become synonymous with modern agriculture range from leaching of pesticides and added nutrients into groundwater or nearby rivers and the simultaneous non-selective killing of pests and beneficial animals like predators of pests. Various governments have taken steps to ban some of the more environmentally damaging pesticides though the problem arises of how to continue to increase crop output whilst the means to protect the crop are diminishing.

There are a few possibilities for more sustainable forms of agriculture. The most ecologically friendly type is one where no synthetic chemicals are used at all. However this

method of farming is extremely labour intensive, requires greater land area and the gains of not using expensive pesticides cannot offset the increase in labour costs.

An alternative possibility is the development of more ecologically friendly pesticides. Organophosphates, carbamates and pyrethroids are the most abundantly used pesticides at present. They act as neurotoxins by disrupting certain aspects of acetylcholine esterase activity or the voltage sensitive sodium channel (Szczepanski, 1990). This limited number of working mechanisms has resulted in a range of insect populations developing a resistance to these compounds and therefore posing a serious limitation to their continuous usage. Khambay and O'Connor (1993) pointed to the development of new insecticides by screening derivatives of known natural products followed by qualitative structure-activity relationship studies.

Crops can be made more resistant by increasing natural defences through careful breeding programmes or by the production of foreign proteins after genetic modification. Potential pitfalls are the loss of positive traits such as good taste and high productivity. Consumers are also less likely to tolerate genetically engineered plants.

For closed systems, such as greenhouses, the use of biological control agents like entomophagous pathogens and predatory or parasitic insects and mites seems to hold some considerable promise. Concern that releasing large numbers of these insects would cause major disruption in agricultural ecosystems may prevent this type of control being applied to open fields (Titchmarsh, 1996).

There may be some advantage to planting different species of plant together in order to reduce the risk of attack from pests. Theunissen *et al.* (1995) investigated the effects of intercropping white cabbage with clovers on pest infestation especially by the cabbage moth (*Mamestra brassicae* L.), cabbage root fly (*Delia radicum* L.) and cabbage aphid

(*Brevicoryne brassicae* L.). The results showed a significant suppression of oviposition and larval populations of various pests. Although no pesticides were used and competition reduced the weight, the quality of cabbages led to a better financial result compared with the monocropped cabbage crop.

One final possibility is perhaps a shift from toxic insecticides to behaviour modifying compounds. The population size of a pest insect in a localised area can be controlled by disrupting the mating process with the use of oviposition deterrents or traps baited with synthetic pheromones. The female oriental fruit moth (*Grapholita molesta*) is a pest of deciduous fruits throughout most of the world. Its favourite host is quince but the greatest economic losses arise from attack on peaches and nectarines, (Summers, 1966). The female produces an attracting pheromone of which the major components are *(Z)-8-* and *(E)-8-*dodecenyl acetates and *(Z)-8*-dodecen-*1*-ol (Cardé *et al.*, 1979). They found that to evoke upwind flight and all of the behaviours leading to courtship all three components had to be present in the right ratios. Field trials in California and Virginia peach orchards with polyethylene dispensers containing synthetic components of the pheromone to disrupt mating have showed that pheromone treatments can reduce the extent of moth damage at harvest (Pfeiffer *et al.*, 1986). Similar mating disruption programmes using synthetic pheromones are used to control the Pink Bollworm (Staten *et al.*, 1987) which is a major pest of cotton plantations in the United States and for the Gypsy Moth (Plimmer *et al.*, 1982) a devastating pest affecting over 500 species of hardwood forest, shade and ornamental trees in Europe and the United States.

In insects, hormones are required to control the different stages in the life cycle from larva to adult. They are required to initiate the metamorphoses that occur during growth as outlined in Figure 2. Juvenile hormones (JH) are only required at the initial changes from

stage to stage whereas the moulting hormones (MH) which control the moulting of the cuticle, are required for every step up until adulthood.

FIGURE 2 OUTLINE OF STAGES IN INSECT DEVELOPMENT

For normal development from larva to adult the two hormones must be present at appropriate, precisely defined amounts and at the right time in the life cycle. Research into disrupting insect growth has led to the isolation of juvenile hormones such as JH0 and JH1 (Harborne, 1988) and the production of juvenile hormone analogues in particular Methoprene (Bowers, 1982) and MV-678 (Schwarz *et al.*, 1974) (Fig. 3).

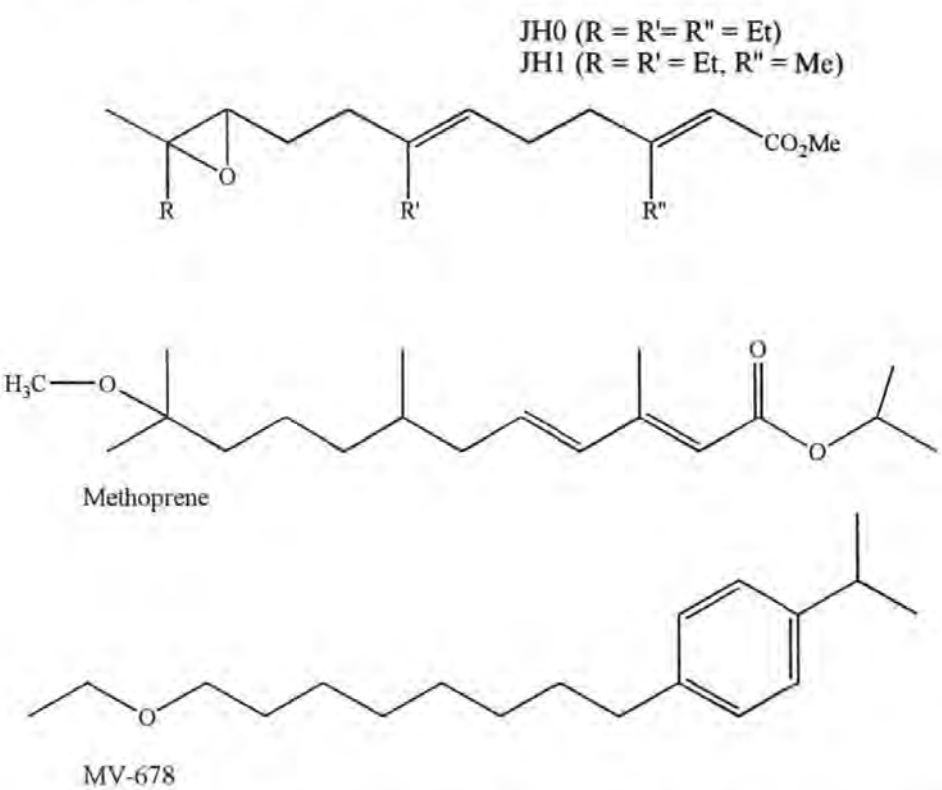
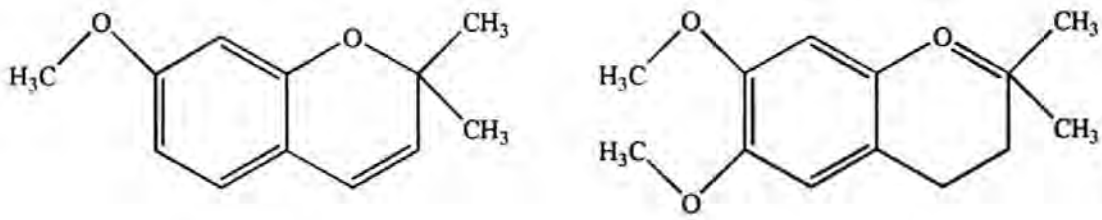


FIGURE 3 TWO JUVENILE HORMONES (JH0 AND JH1) AND TWO HORMONE ANALOGUES (METHOPRENE AND MV-678)

These analogues of the natural juvenile hormones penetrate the cuticle of the insect and produce a lethal disruption of the development process. However, this strategy is limited in that the synthetic analogues will only disrupt development at limited stages in the life cycle. This problem was overcome with the discovery of synthetic preocenes (Fig. 4) and the production of insect growth regulators TH-6038 and TH-6040 (Oliver *et al.*, 1976) (Fig. 5).The former interfere with hormone biosynthesis in such a way that one or more larval stages are missed out resulting in imperfect adults (Harborne, 1988)



Preocene 1 Preocene 2

FIGURE 4 TWO ANTI-JUVENILE HORMONE COMPOUNDS

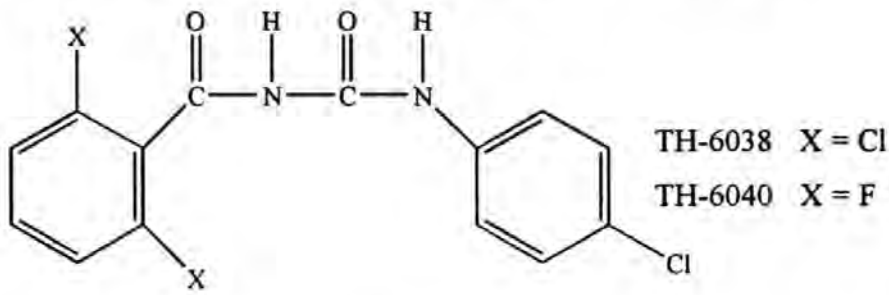


FIGURE 5 TWO INSECT GROWTH REGULATORS

There has also been considerable research into the naturally occurring insect antifeedant molecules. For example, the powerful triterpenoid antifeedant azadirachtin which is produced by the neem tree (*Azadirachta indica* L.) as part of its insect defence against desert locust in Africa (Harborne, 1988) and is readily available from the seeds. The structure of azadirachtin (Bilton *et al.*, 1987) is shown in Figure 6.

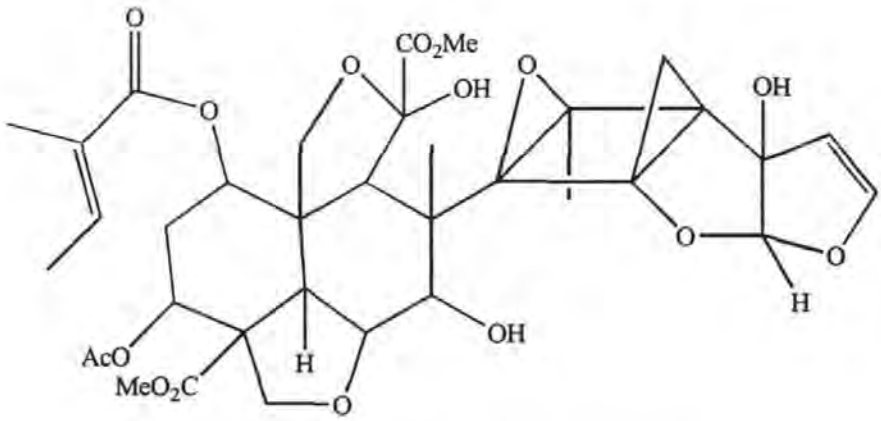


FIGURE 6 STRUCTURE OF AZADIRACHTIN

A more controlled application of pesticides may prove to be a suitable compromise between a decrease in the use of expensive and environmentally damaging chemicals and an increase in crop quality. Ester *et al.* (1994, 1997) studied the protection against cabbage root fly larvae achieved in cauliflower seeds and Brussels sprouts seeds by film-coating with insecticide. The efficacy of formulations of Isofenphos, Chlorpyrifos and Fonofos at three different concentrations were compared with a conventional post-planting treatment. Film-coating with Fonofos was less effective in controlling cabbage root fly larvae than post-planting treatment. Chlorpyrifos and Isofenphos were consistently found to give as good control as a post-planting treatment of each plant though the amount of insecticide used was much less in each case.

Attractants, either pheromones or semiochemicals can be used for monitoring the population size of pests and therefore making it possible to predict whether the crop will suffer damage. An example of this is the recent work at the University of Plymouth in collaboration with the University of the West of England and W. H. Knights and Son Limited in which the study of the behavioural response of the cabbage root fly to a group of host plant volatiles known as isothiocyanates, has led to the development of a commercial monitoring trap. The trap, marketed as BrassicEye™ uses isothiocyanates to specifically

attract gravid female cabbage root flies. The farmer checks the trap periodically and, based on the number of flies caught, can determine whether it is necessary to spray the crop. The need to spray the crop continually with expensive and ecologically unfriendly pesticides throughout the breeding season of the fly is then reduced.

1.3 THE CABBAGE ROOT FLY

The cabbage root fly, *Delia radicum* L. (Diptera; Anthomyiidae) is a major pest of cabbage, cauliflowers, Brussels sprouts, swedes and other cultivated brassicas (Coaker and Finch, 1971) throughout North America and Europe. It is most prevalent in Britain on cauliflowers and summer cabbage. Roots are attacked by the larvae at all stages of plant development but young plants usually sustain the most serious damage and can be killed if attacked severely in the seedbed or shortly after transplanting. Spring cabbage and the buttons of Brussels sprouts can be directly affected in late summer and autumn though generally the most serious damage usually occurs in late April and May, and again in July.

1.3.1 Appearance and Life Cycle

The fly (Plate 1) is approximately 6 mm in length and grey in colouration. There are two generations each year, with a third generation occurring in some areas though it is dependent on the ambient temperature. In most parts of the UK the first generation emerges in late April and May at the same time that the umbellifer weed Cow Parsley (*Anthriscus sylvestratis*) comes into flower. The second generation appears in late June and July with the third generation from August onwards. The later generations tend to overlap and so in some areas flies are found continuously from July through to September.

PLATE 1 FEMALE CABBAGE ROOT FLY (X 4)

The female generally lays her batch of 50 to 60 eggs on or just below the soil surface in cracks or between soil particles close to the main stem of the plant (Traynier, 1967). The eggs may also be laid on the foliage or other parts of the plant like Brussels sprout buttons. The first eggs hatch three to seven days after laying depending on the temperature.

The larvae are white or cream coloured (Plate 2) and about 1 mm in length. They feed on the root tissues close to the tap root. Some can be found in the stem, growing points and midribs of the leaves as well as sprout buttons. After about three weeks, when approximately 8 mm in length and fully grown, the maggot leaves the plant and moves through the soil down and away from the roots. Here it changes into a pupa inside a brown pupal case from which it emerges as an adult fly. Later generations overwinter in the pupal stage to emerge as an adult the following spring.

PLATE 2 CABBAGE ROOT FLY LARVA (X 10)

1.3.2 Crop Damage

Plants that are attacked by the larvae in the early stages of growth, or just after transplantation, often wilt and die. This is a direct result of a badly damaged tap root, devoid of any fibrous roots which eventually rots or withers. Occasionally the plant may grow some new roots from the base of the stem although it's growth will be severely stunted and the plant will be of little or no commercial value. Less severe attacks (Plate 3) will stunt plants to varying degrees and, for example, will reduce the size of cauliflower curds. Mature plants can withstand a certain level of attack without showing any obvious signs of damage but the yield may still be reduced.

PLATE 3 DAMAGED CABBAGE ROOT

For swede, kale, turnip and radish even superficial damage by the cabbage root fly larvae to the roots, bulb or crown will effect the commercial value of the crop. Although the weight is not affected by such light damage the majority of consumers will not tolerate the resulting less than perfect physical appearance. Larvae that emerge from eggs laid in the folds of the enveloping leaves of Brussels sprout buttons burrow into the sprout and remain undetectable until they leave to pupate in the soil. Some larvae can, therefore, be harvested

with the sprouts. In crops grown for quick freezing this can cause quality problems even when only a few per cent of the buttons are affected.

1.4 OVIPOSITION BEHAVIOUR

One area of the expanding field of insect-plant relationships focuses on the process by which pytophagous insects find and accept their host plants. The sensory cues that elicit oviposition clearly play an important part in the survival of most phytophagous insects. The oviposition step is crucial to many species because the hatching larvae are relatively immobile and therefore depend on the choice of food plant by the adult female.

It is known that plant compounds in the leaf surface are involved in insect-host plant relationships (Städler and Roessingh, 1990) and that for many insects the choice of host-plant is with the ovipositing female (Städler, 1992). The cabbage root fly is no exception.

Many aspects of the cabbage root fly's behaviour have been well investigated and documented by several authors (Nottingham, 1988; Städler and Schöni, 1990; Kostál, 1992a, 1992b, 1993a, 1993b).

1.4.1 Host Plant Finding

Much of host-plant finding depends upon the insect being able to recognise characteristic chemical odours produced. Early workers assumed that orientation to an odour was by sensing a concentration of odour molecules and following this from an area of low molecular density to an area of high molecular density i.e. the source (chemotaxis). Gradients will, however, only operate over a small distance in open air because of disruptive air flows due to temperature differences that exist amongst objects. In open air it is air-turbulence rather than diffusion that mainly determines the distribution of odour molecules (Bossert and Wilson, 1963).

Host plant volatiles have been shown to play an important part in the location of brassica plants by the female cabbage root fly (Hawkes, 1974; Hawkes, 1975). Wind tunnel experiments have shown that the allyl isothiocyanate component of host plant odour acts as an attractant (Hawkes and Coaker, 1979). Gravid female cabbage root fly orientate upwind (anemotaxis) in the presence of these host plant volatiles and fly in a series of short flights to the source of the volatile components. Anemotaxis while on the ground involves orientation into the wind prior to take off using antennal anemo-receptors to detect wind direction directly by mechanical effects (Kennedy, 1977). If already in flight, optomotor anemotaxis occurs. The insect uses its movement relative to a fixed visual reference to assess effects of wind drift in its airspeed and course. Therefore wind speed and direction can be calculated and the insects airspeed and course set accordingly (Kennedy, 1977). The loss of the odour stimulus during an upwind flight causes the fly to land or turn downwind, circling and landing at frequent intervals, until the odorous air is encountered once again when upwind flight then resumes. As the fly nears the host-plant source the odour plume becomes less diffuse, the distance covered in each flying step increases and the insect's speed decreases (Nottingham and Coaker, 1987).

Aluja *et al.* (1993) studied the effect of wind speed on female *Rhagoletis pomonella* Walsh (apple maggot fly) responses to synthetic host apple volatiles in a wind tunnel. Anemotaxic fly movement was shown to decrease as the speed of odour carrying wind increased from 0 m s^{-1} to 0.8 and 1.6 m s^{-1}. When air was odour-free, flies exposed to 1.6 m s^{-1} wind moved downwind.

1.4.2 Landing

Landing on a plant is the last stage in the orientation process and may be triggered by either chemical or physical cues but most often by a combination of the two. Landing

frequencies in a population may change seasonally with host abundance or quality (Courtney and Fosberg, 1988) or depend on the relative abundance of a preferred host (Rausher, 1979). Also, in some species previous experience can increase accuracy of ovipositing females (Vet and Papaj, 1992; Papaj *et al.*, 1992).

Colour probably plays less of a role in the behaviour of night flying insects although the Yucca Moth (*Tegeticula maculata*) selects inflorescences of a specific size and colour for oviposition (Ahmad, 1983). The role of volatile chemical components of plants in eliciting landing has been suggested although the observed effects could also be attributed to attraction by other factors. The Black Swallowtail Butterfly (*Papilio polyxenes*) laid more eggs on artificial plants that were treated with carrot volatiles in addition to contact stimulants than on models that lacked the volatiles (Feeny *et al.*, 1989). It was suggested that this increase in the number of eggs could be attributed to an increased frequency of landing on the volatile-treated models. Furthermore, volatiles from a non-host plant, cabbage, inhibited landing by the female butterflies. Electroantennograms showed that the antennal receptors responded more to the carrot volatiles than to the cabbage volatiles (Baur *et al.*, 1992). Cabbage volatiles, such as isothiocyanates, have been shown to be attractive to the cabbage root fly and other insects including the weevil *Listroderes obliqueus* Klug. (Matsumoto, 1970) and the Flea Beetle (*Psylliodes chrysocephala* L.)(Queinnec, 1967).

It has been shown that landing by the cabbage root fly is influenced predominantly by visual stimulation (Hawkes and Coaker, 1979). Kostál (1993b) used various plant models in both choice and no-choice tests in the laboratory to assess landing and oviposition preferences of the cabbage root fly. The main factor governing the site most suitable for landing was the conspicuousness of the object and not its shape. The dominant role of

contact chemical stimuli in host-plant acceptance was reconfirmed but only a combination of physical and chemical stimuli appeared capable of eliciting normal oviposition. The combination of contact chemical stimuli and the presence of a stem on the test model had a synergistic effect on the numbers of eggs laid in both choice and no-choice situations. In choice bioassays, female cabbage root flies distinguished between models of different shapes, heights and sizes, though the size and shape of the models appeared to be perceived in part after the fly had landed. It has been shown that in the absence of chemical stimuli, females lay the most eggs around the base of bright green or yellow models that have a stem, vertical folds and covered in a thin uniform layer of paraffin wax (Roessingh and Städler, 1990)

A further study by Kostál and Finch (1994) of the behaviour of female cabbage root flies during host-plant selection in the laboratory used brassica plants growing against backgrounds of bare soil, clover, grass, peas and green and brown paper. Gravid females landed about twice as often on plants growing in bare soils and brown paper than on plants growing amongst non-host plants or green paper. Surrounding a brassica plant with a diverse background altered the behaviour of the flies so that flights around the host-plant are replaced with short hops between visually stimulating, i.e. (green) objects nearby. The loss of contact and recontact with the host plant then prevents the females from accumulating sufficient contacts with the host plant to be stimulated to lay.

1.4.3 Contact Evaluation

Once a gravid female insect has landed on a host-plant, evaluation of the array of sensory information available at the plant surface proceeds very rapidly. Combinations of physical and chemical factors are involved in this evaluation.

Plant surface texture seems to be more important for nocturnal insects, such as moths than for diurnal species. The majority of moths appear to prefer hairy or rough surfaces such as the shaggy substrates preferred by *Catocala* moths (Gall, 1990). Callahan, (1957) suggested that the hairs allow the females to maintain a better footing on the leaf during oviposition, and that hairy leaves may retain eggs better than smooth leaves.

Certain aspects of butterfly behaviour are consistent across the families. The "drumming" reaction, first described by Ilse (1937), appears in some form in all the species that have been observed. In each case the forelegs move rapidly so that the terminal segments of the tarsi drum against the leaf surface. Also, the drumming is often accompanied by wing fluttering. It has been suggested that this action may waft released volatiles toward antennal receptors. Several workers have performed experiments to demonstrate the involvement of tarsal chemoreceptors in host recognition for oviposition (Myers, 1969; Ichinoisé and Honda, 1978; Calvert and Hanson, 1983). However, many of these studies also point out that several sensory organs are involved in the whole process and that redundant systems may exist. It has been observed that female butterflies probe the surface with their proboscis (Platt, 1979), and contact chemoreceptors have been found on the proboscis of some lepidopterans (Frings and Frings, 1949).

Städler and Schöni (1990) classified a number of behaviour patterns exhibited by female cabbage root flies after landing (Fig. 7). Short visits of between 1 and 30 seconds were classed as pattern A. Resting with cleaning of the tarsi, wings and head was classed as pattern B. Repeated proboscis contact whilst running over the leaf was described as exploration behaviour and classified as pattern C. Some flies were observed to return from pattern C to pattern B though after a period of variable duration, the exploration behaviour (pattern C) would begin again or the fly would leave the leaf. Sufficiently stimulated flies

would undergo the whole sequence of preoviposition (B-C), oviposition, and postoviposition behaviour (D-H). The sequence could be interrupted by departure from the leaf at any of the stages described. The flies were also observed to return to a previously displayed behaviour, therefore repeating some steps in the sequence.

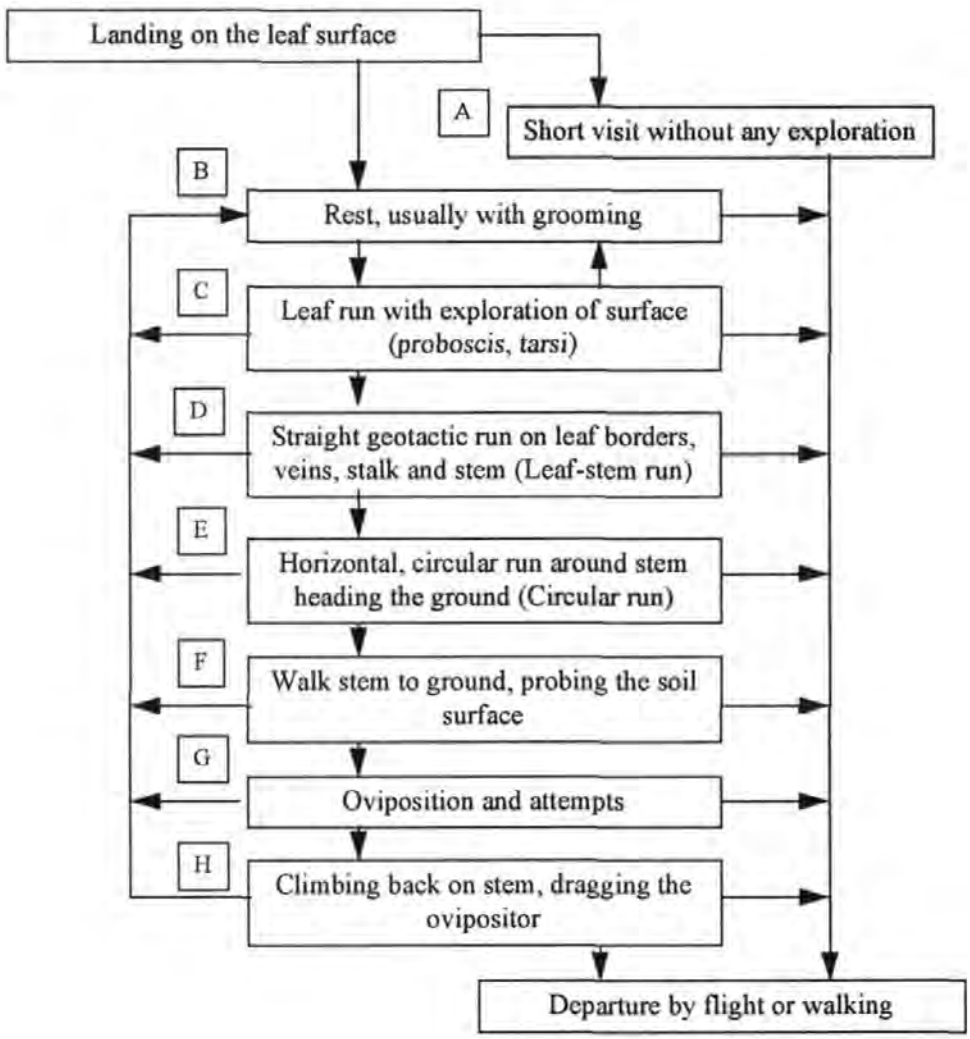


FIGURE 7 ETHOGRAM OF THE OVIPOSITION BEHAVIOUR OF THE CABBAGE ROOT FLY (STÄDLER AND SCHÖNI, 1990)

Traynier (1967) observed that more eggs were laid between soil particles of two different sizes 0.42-0.85 mm and 1.00-1.75 mm in diameter. It seemed that these soils provided a high density of cracks which permitted the entry of the extended ovipositor. In the laboratory equal numbers of eggs were laid between smooth glass spheres, 1.5-2.0 mm

in diameter and similar spheres that had been roughened by grinding with silicon carbide paper. Further observations showed that the optimum physical requirements for oviposition are not only cracks of the same size as the ovipositor, but also irregularities within the cracks against which eggs may be deposited.

1.4.3.1 Chemical Stimulation

The number of compounds identified as oviposition stimulants or as components of a stimulatory blend has grown substantially in the last 20 years. Studies have provided information about the involvement of different chemical classes of compounds associated with different host plants of specialist insects. The buckeye butterfly (*Junonia coenia*) is one example of a specialist that appears to depend on the typical secondary plant chemicals of its host plants as cues for oviposition. This insect is a specialist of plants containing iridoid glycosides. Two prominent iridoid glycosides, aucubin and catapol, present in the host, *Plantago lanceloata*, were tested and found to be active as oviposition stimulants (Pereya and Bowers, 1988). Similarly sinigrin (prop-*2*-enylglucosinolate), a characteristic compound present in hosts of the diamond back moth (*Plutella xylostella)* stimulated oviposition by gravid female moths (Renwick and Radke, 1987).

Sinigrin is one of a family of compounds known as glucosinolates. These glucosides are the precursors of the volatile isothiocyanates and are found in varying numbers and concentrations throughout the order Capparales. Glucosinolates are known to be important stimuli for many other brassica feeding insects (Städler, 1992).

It is known that the presence of sinigrin (David and Gardiner, 1962) or the presence of cabbage leaf extract which contains glucosinolates (Rothschild and Schoonhoven 1977) will stimulate egg laying in *Pieris brassicae* and *Pieris rapae*. Sang *et al.* (1984) demonstrated that most predominant glucosinolates in brassica foliage were the indolyl

glucosinolates. Of these, glucobrassicin (indol-*3*-ylmethylglucosinolate) has been shown to be a potent natural egg laying stimulant for the cabbage butterflies *Pieris rapae, Pieris brassicae* and *Pieris napi oleracea* (Traynier and Truscott, 1991; Huang *et al.*, 1994). Non-indolyl glucosinolates have also been compared as oviposition stimulants for cabbage butterflies (Renwick and Chew, 1994; Huang *et al.*, 1994) and while eliciting an oviposition response none could match the potency of glucobrassicin.

Contact chemoreception plays a decisive role in the oviposition behaviour of the cabbage root fly which was shown to be able to differentiate between glucosinolates. As with cabbage butterflies the indole glucosinolates stimulated oviposition behaviour more than aromatic or aliphatic glucosinolates (Schöni *et al.*, 1987; Städler and Schöni, 1990; Roessingh *et al.*, 1992). Sinigrin was tested in solution by Traynier (1967) but even at 2500 mg dm^{-3} it was a weaker stimulant than freshly pressed sap from the hypocotyl of *Brassica napus*. A total of five glucosinolates (in combination with allyl isothiocyanate) were tested by Nair and McEwen (1976) but although high concentrations were used, oviposition stimulation was low in comparison with sliced rutabaga (*Brassica napobrasica* Mill.) Nair and McEwen also showed that contact with glucosinolates is necessary for ovipositon, the presence of the host plant volatile isothiocyanates being insufficient alone, a fact that has since been corroborated by Städler (1992).

Roessingh and co-workers (1992) showed that the D sensilla on segment 3 and 4 of the tarsus of *Delia radicum* females contain a sensitive receptor cell for glucosinolates. In contrast, the receptor cells of the D sensilla on the other segments did not respond in a dose dependent way to these compounds. The glucosinolate receptors were found to be especially sensitive to glucobrassicin, gluconasturtiin (phenethylglucosinolate) and glucobrassicanapin (pent-*4*-enylglucosinolate). The observed order of effectiveness in

behavioural and electrophysiological terms corresponded well. However the outstanding behaviour of glucobrassicin could not be correlated with the electrophysiological data. It was suggested that this could be due to the fly having additional glucosinolate receptors with a higher specificity for glucobrassicin. Possible sites could be the numerous tarsal A sensilla with glucosinolate receptors (Städler, 1978).

Simmonds *et al.*, (1994) studied the neural mechanisms involved in the behaviour of the turnip fly (*Delia floralis* Fallen). Electrophysiological responses were obtained from the long contact sensilla on the labellum as well as type A and D sensilla on the prothoracic and mesothoracic tarsi. Of the eleven glucosinolates tested the flies were most responsive to glucobrassicin, gluconapin (but-*3*-enylglucosinolate) and glucobrassicanapin. The type D sensilla were more sensitive to the glucosinolates than either the type A tarsal sensilla or the labellar sensilla. Contact chemoreceptor sensilla were also located on the proboscis of *Delia floralis*. For the corresponding sensilla of the cabbage root fly only preliminary, negative results, obtained with sinigrin, are available (Städler, 1978).

The comparison of glucosinolate contents of different species of crucifer with oviposition on these plants (Nair *et al.*, 1976) found no correlation. Despite this it was concluded that glucosinolates could be important for oviposition. Based on difference in the activity of some glucosinolates tested (Nair and McEwan, 1976) it was suggested that the actual range of compounds was of greater importance for oviposition preference than the total glucosinolate content.

Roessingh *et al.* (1992) found a significant correlation between the overall length of the side chain of the glucosinolate molecule and biological activity. Interestingly, however, the most stimulating fraction of plant surface extract contained no glucosinolates. It has been shown that C sensilla on segment 5 of the tarsus of both the cabbage root fly and

turnip fly are sensitive to this non-glucosinolate oviposition stimulant known as CIF (cabbage identification factor) although the chemosensory response of *Delia floralis* was less pronounced than the response of *Delia radicum* (Roessingh *et al.*, 1992; Baur *et al.*, 1996).

1.4.3.2 Chemical Deterrents

Few attempts to explain host specificity of insects on the basis of oviposition deterrents have been made. The possible involvement of deterrents in host selection by the cabbage butterfly, *Pieris rapae*, which is a specialist on brassica and related plants has been studied in some detail. Examination of one unacceptable plant, *Erysiumum cheiranthoides*, has led to the isolation and identification of cardenolides that can explain the deterrent activity (Sachdev-Gupta *et al.*, 1990). Two of the three major cardenolides found in the active fraction showed biological activity. The deterrent compounds, ersimoside and erychroside, both have strophanthidin as the aglucone, but differ in the attached sugars. Commercially available cymarin, which also has a strophanthidin aglucone was active but other cardenolides had little or no deterrent activity.

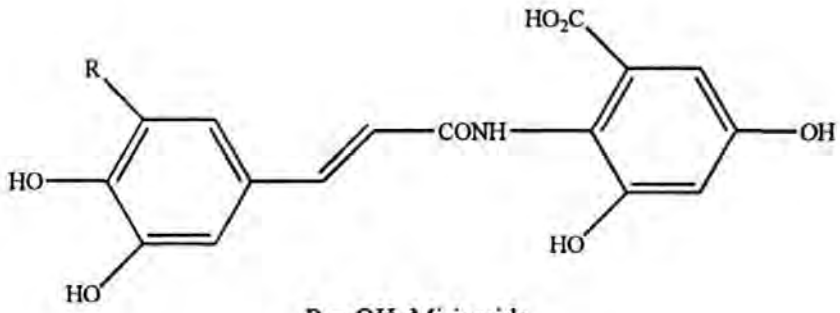
Gravid female *Pieris rapae* also reject *Iberis amara* as a host plant and two curcubitacin glycosides can account for the activity (Huang *et al.*, 1993). A detailed study of oviposition preferences of *Pieris rapae* and *Pieris napi oleracea* for a series of 11 potential host plants demonstrated the combined role of deterrents and stimulants in determining host ranges of these butterflies (Hayes, 1981).

The cabbage root fly is deterred from laying its eggs in the soil alongside plants contaminated with the frass of caterpillars of the garden pebble moth (*Evergestis forficalis* L.) (Jones and Finch, 1987). The deterrent effect is independent of the plant species on which the caterpillars have been feeding (Jones and Finch, 1987) but arises because the

caterpillars excrete in their frass a chemical isolated and identified as sinapic acid, a phenolic acid (Jones *et al.*, 1988). Cole and co-workers (1989) selected 11 carboxylic acids and tested them to determine which part of the sinapic acid molecule is responsible for deterring the cabbage root fly from laying its eggs. The deterrent effect was only obtained with compounds containing at least one carboxylic group in the molecule. The aliphatic acids were as deterrent as the aromatic acids and the inclusion of two carboxylic acid groups in the molecule did not increase activity. None of the acids matched sinapic acid for its potency.

A blend of three monoterpenes (3-carene, limonene and *p*-cymene) was found by Ntiamoah *et al.* (1996) to be a major oviposition deterrent for the onion fly (*Delia antiqua* L.). Oviposition by the cabbage root fly was deterred significantly by the same ternary mixture. Increasing the complexity of the mixture by adding terpinolene, γ-phellandrene and myrcene was shown to increase the deterrence markedly (Ntiamoah and Borden, 1996).

After ovipositing, females of some herbivorous insect species are known to deposit a marking substance on or near the eggs to discourage visits by other conspecifics. Three such oviposition-deterring markers have been chracterised by extracting the eggs of *Pieris brassicae*, the large white butterfly. The compounds as shown in Figure 8 are cinnamic acid substituted hydroxybenzoic acids: miriamide, miriamide 5-glycoside and 5-dehydroxymiriamide (Blaakmeer *et al.*, 1994).



R = OH, Miriamide

R = OGlc, Miriamide 5-glucoside

R = H, 5-dehydroxymiriamide

FIGURE 8 **STRUCTURES OF THREE OVIPOSITION-DETERRENT COMPOUNDS FOR THE LARGE WHITE BUTTERFLY (BLAAKMEER *ET AL.*, 1994)**

Braven and co-workers (unpublished) have established evidence to suggest the presence of chemical markers on the eggs of the cabbage root fly which have an oviposition deterrent affect on other gravid cabbage root fly females. The compound or compounds responsible for this deterrence have yet to be identified.

Once the basic chemical and physical requirements for oviposition by an insect have been met, the probability that an egg will actually be laid still depends on the physiological state of that insect (Städler, 1992).

1.5 GLUCOSINOLATES

1.5.1 Structure and Distribution

Glucosinolates are the most important secondary plant compounds in the *Brassicaceae* family. Their presence in agricultural crop plants is especially significant because of the potentially harmful effects of their breakdown products to livestock consuming such crops and because of their role in insect or pathogen - plant interactions (Chew, 1988).

Glucosinolates occur in only 11 families of dicotyledonous plants mainly in the order Capparales and principally in the families Brassicaceae, Resedaceae and Capparidaceae, although their sporadic presence in other families such as Caricacae, Salvadoracae and Tropæolaceae has been reported (Larsen, 1981). The use of many of these plants as vegetables, condiments and medicines originated many centuries ago (Fenwick *et al.*, 1983); undoubtedly one of the primary reasons for this culinary and medical interest was the pungent, sharp flavour and irritant properties of the plants when crushed. The first concentrates of these volatile substances were derived from mustard plants and thus the name "mustard oils" was given to these early concentrates. In 1830 it was found that the volatile oils were not present in the plants as such but derived from involatile precursors (Roubiquet and Boutron, 1830). By 1840 two of these mustard oil glycosides had been isolated and named as sinigrin and sinalbin. Fifty years later Gadamer (1897a, 1897b) suggested a general structure (Fig. 9) based on a detailed investigation of the observed breakdown products. However in spite of the fact that a number of chemical properties peculiar to this class of glycoside could not be accounted for by his formula it was not until 1956 that Ettlinger and Lundeen proposed the correct structural formula for sinigrin (prop-*2*-enylglucosinolate) and sinalbin (*p*-hydroxybenzylglucosinolate). This was later confirmed by synthesis (Ettlinger and Lundeen, 1957) and x-ray diffraction studies (Waser and Watson, 1963; March and Waser, 1970). Of the hundred or more glucosinolates that have been isolated and characterised all possess the same skeleton (Fig. 10) and differ only in the side chain R.

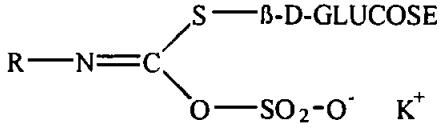


FIGURE 9 **GENERAL STRUCTURE OF GLUCOSINOLATES (GADAMER, 1897)**

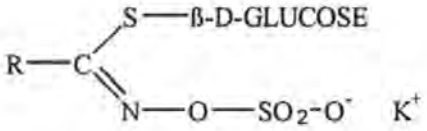


FIGURE 10 GENERAL STRUCTURE OF GLUCOSINOLATES (ETTLINGER AND LUNDEEN, 1957)

Most naturally occurring glucosinolates are difficult to crystallise and fewer than half have been characterised as crystalline glucosides or glucoside derivatives. Most glucosinolates are isolated as sodium or potassium salts although hydroxy-*p*-benzylglucosinolate is unique in the sense that it has sinapine, a rather widely distributed quaternary base, as its cationic moiety (Kjær, 1960). Some glucosinolates such as benzylglucosinolate are isolated as tetramethylammonium salts which have been shown to possess good crystallisation properties (Ettlinger and Lundeen, 1957). The structures of the other glucosinolates have been inferred on the basis of their chemical and enzymatic degradation products. Table 2 shows common glucosinolate side chains and their main source of isolation.

SIDE CHAIN	TRIVIAL NAME	MAIN PLANT SOURCE
Pent-*4*-enyl	Glucobrassicanapin	*Brassica napus* L.
Indol-*3*-ylmethyl	Glucobrassicin	*Brassica oleracea* L.
But-*3*-enyl	Gluconapin	*Brassica campestris* L.
2-Phenylylethyl	Gluconaturtiin	*Nasturtium offficinale* R.
4-Methylsulphinyl butyl	Glucoraphanin	*Brassica oleracea* L.
4-Methylsulphinyl-*3*-butenyl	Glucoraphenin	*Raphanus sativus*
Benzyl	Gluotropæolin	*Tropæolum majus* L.
2-Hydroxybut-*3*-enyl	Progoitrin / Glucorapiferin	*Brassica vapa*
Hydroxy-*p*-benzyl	Sinalbin	*Brassica napus* L
Prop-*2*-enyl	Sinigrin	*Brassica nigra* Koch

TABLE 2 SIDE CHAINS OF COMMON GLUCOSINOLATES AND THEIR MAIN SOURCES

The nomenclature used to designate individual members of this class of thioglucosides before 1961 was based on a system of trivial names. Apart from the first glucosides, sinigrin and sinalbin, the trivial names were derived from the Latin name of the plant from which the glucoside was first isolated, prefixed with 'gluco' and suffixed with 'in', e.g. glucotropæolin from the *Tropæolium* species. Ettlinger and Dateo (1961) introduced a semi-systematic method of nomenclature in which the anion of the parent salt (R=H) was designated by the term 'glucosinolate' and the chemical group (R) is used as the prefix, e.g. glucotropæolin = benzylglucosinolate.

Although over 100 different glucosinolates have been described (McDanell *et al.*, 1988) it was generally thought that individual species contained relatively few, at least in high abundance. Grob and Matile (1980) studied the hydrolysis products of horseradish following controlled hydrolysis with exogenous myrosinase using GLC-MS and found structural evidence to suggest the presence of 30 glucosinolates. The concentrations of glucosinolate vary throughout the plant, with the seeds and other areas of rapid tissue growth such as the roots and shoots containing more than the other vegetative tissues. Cabbage heads may contain 0.121 mmoles of glucosinolate per 100g fresh material, whereas deffated cabbage seeds contain 18 mmoles per 100g (Tookey *et al.*, 1980). *Brassica napus* (swede) may typically contain 10 mmoles per 100 g in the seeds, while leaf concentrations of 0.1 - 0.2 mmoles per 100 g are common. Different plant parts contain not only different glucosinolate concentrations but also possess differing profiles of individual glucosinolates (Sang *et al.*, 1983). Tables 3, 4 and 5 show the main glucosinolate components in the seeds, leaves and roots of 3 brassica species.

SEED	LEAF	ROOT
Prop-*2*-enyl	Prop-*2*-enyl	Prop-*2*-enyl
4-Hydroxyindol-*3*-ylmethyl	*4*-Methoxyindol-*3*-ylmethyl	*2*-Phenylethyl
2-Phenylethyl	*4*-Hydroxyindol-*3*-ylmethyl	*4*-Methoxyindol-*3*-ylmethyl
	2-Phenylethyl	*4*-Hydroxyindol-*3*-ylmethyl
	Indol-*3*-ylmethyl	1-Methoxyindol-*3*-ylmethyl

TABLE 3 **MAIN GLUCOSINOLATE COMPONENTS FOUND IN THE SEED, LEAF AND ROOT OF MUSTARD (*Brassica junacea*) IN DESCENDING CONCENTRATION ORDER**

SEED	LEAF	ROOT
2-Hydroxybut-*3*-enyl	*2*-Hydroxybut-*3*-enyl	*2*-Hydroxybut-*3*-enyl
But-*3*-enyl	Indol-*3*-ylmethyl	*1*-Methoxyindol-*3*-ylmethyl
4-Hydroxyindol-*3*-ylmethyl	*2*-Hydroxypent-*4*-enyl	*4*-Methoxyindol-*3*-ylmethyl
Indol-*3*-ylmethyl	Pent-*4*-enyl	Indol-*3*-ylmethyl
2-Hydroxypent-*4*-enyl	But-*3*-enyl	*4*-Methylthiobutyl
Pent-*4*-enyl	*4*-Methoxyindol-*3*-ylmethyl	*4*-Methylsulphinylbutyl
	1-Methoxyindol-*3*-ylmethyl	*2*-Phenylethyl
	4-Hydroxyindol-*3*-ylmethyl	*4*-Hydroxyindol-*3*-ylmethyl

TABLE 4 **MAIN GLUCOSINOLATE COMPONENTS FOUND IN THE SEED, LEAF AND ROOT OF SWEDE (*Brassica napobrassica*) IN DESCENDING CONCENTRATION ORDER**

SEED	LEAF	ROOT
2-Hydroxybut-3-enyl	Indol-3-ylmethyl	Indol-3-ylmethyl
Prop-2-enyl	2-Hydroxybut-3-enyl	3-Methylthiopropyl
4-Methylthiobutyl	4-Methoxyindol-3-ylmethyl	4-Methoxyindol-3-ylmethyl
But-3-enyl	Prop-2-enyl	2-Phenylethyl
4-Hydroxyindol-3-ylmethyl	4-Hydroxyindol-3-ylmethyl	4-Methylthiobutyl
	4-methylsulphinylbutyl	Prop-2-enyl
	1-Methoxyindol-3-ylmethyl	1-Methoxyindol-3-ylmethyl
		4-Hydroxymethylindol-3-ylmethyl

TABLE 5 MAIN GLUCOSINOLATE COMPONENTS FOUND IN THE SEED, LEAF AND ROOT OF CABBAGE (*Brassica oleracea*) IN DESCENDING CONCENTRATION ORDER

The types of glucosinolates present in a particular plant is controlled genetically and has been used as a chemotaxonomic parameter and in cultivar identification within species (Adams and Vaughan, 1989). No authenticated member of the *Brassicaceae* has been found to be devoid of glucosinolates and methylglucosinolate is found only within the family *Capparaceae*. There are also a number of environmental factors which affect glucosinolate profiles and concentrations. An increase in sulphate in the soil will increase the concentrations of glucosinolate in both the seeds and the leaves (Mailer, 1989), while an increase in soil nitrogen has the opposite effect of reducing glucosinolate concentrations (Heaney *et al.*, 1983). Herbivore damage may increase glucosinolate concentrations possibly as a measure to prevent further herbivory (Birch *et al.*, 1990).

The function of glucosinolates in plant metabolism is still not fully understood. The metabolic costs involved in producing them plus their rapid turnover in plant tissues suggests an important adaptive function. There is evidence to suggest that glucosinolates serve to protect the plant against herbivore damage similar to other secondary metabolites

such as the pyrrolizidine alkaloids. Tsao and co-workers (1996) reported insecticidal properties of glucosinolates extracted from Crambe (*Crambe abyssinica*) with respect to mosquito (*Aedes aegypti* L.) larvae, house fly larvae (*Musca domestica* L.) and the western corn root worm larvae (*Diabrotica virgifera virgifera* LeConte.). Glucosinolates and their break down products have been shown to have anti-microbial and anti-fungal properties (Mithen *et al.*, 1986; Mithen, 1992) which may have a role in plant-pathogen resistance.

Insects in addition to the cabbage root fly have adapted to respond positively to glucosinolates; *Pieris brassicae* L. and *Delia floralis* (Fallen) adults oviposit with great discrimination on glucosinolate containing host plants (Roessingh *et al.*, 1992; Simmonds *et al.*, 1994).

1.5.2 Biosynthesis

There is a similarity between the carbon skeleton of 'common' amino acids and some glucosinolates side chains, a fact which lead to the suggestion that amino acids may be the natural progenitors of the aglucone moiety (Kjær, 1954; Ettlinger and Lundeen, 1956). The first published report on the biosynthesis of a glucosinolate (Kutácek *et al.*, 1962) demonstrated that [*3*-^{14}C]tryptophan was converted into indol-*3*-ylmethylglucosinolate (glucobrassicin). Both [*3*-^{14}C]tryptophan and indol-*3*-yl[*1*-^{14}C]acetonitrile were fed to the cabbage and chromatograms of plant extracts showed the presence of radioactive glucosinolate derived from the amino acid but not from the nitrile. In similar experiments Underhill and co-workers (1964) demonstrated that benzylglucosinolate (glucotropæolin) was biosynthesised from the amino acid phenylalanine. Furthermore, when doubly labelled L-[^{14}C,^{15}N]phenylalanine was fed to *Tropæolum majus* L. it was found that the aglucone moiety of the resulting glucosinolate possessed the same ^{14}C/^{15}N ratio as the amino acid (Underhill and Chisolm, 1964). From this it was established that the amino nitrogen and

carbon skeleton of l-phenylalanine, except for the carboxyl carbon, were incorporated intact (Fig. 11), therefore all intermediates between amino acids and the glucosinolate are nitrogenous.

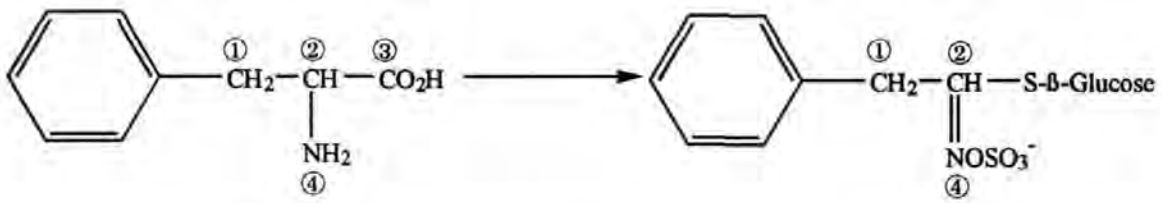


FIGURE 11 CONVERSION OF LABELLED PHENYLALANINE TO BENZYLGLUCOSINOLATE

The fact that the structures of many glucosinolates such as prop-*2*-enylglucosinolate (sinigrin) and the aliphatic glucosinolates in rapeseed were not related in an obvious manner to commonly occurring amino acids suggested that a different biosynthetic pathway existed for their formation. Studies have shown, however, that they are in fact derived from amino acids which are first modified by chain extension, analogous to that seen in the formation of leucine from valine (Fig. 12), to produce α-amino acid homologues whose carbon skeleton and amino nitrogen are specifically incorporated into the glucosinolates.

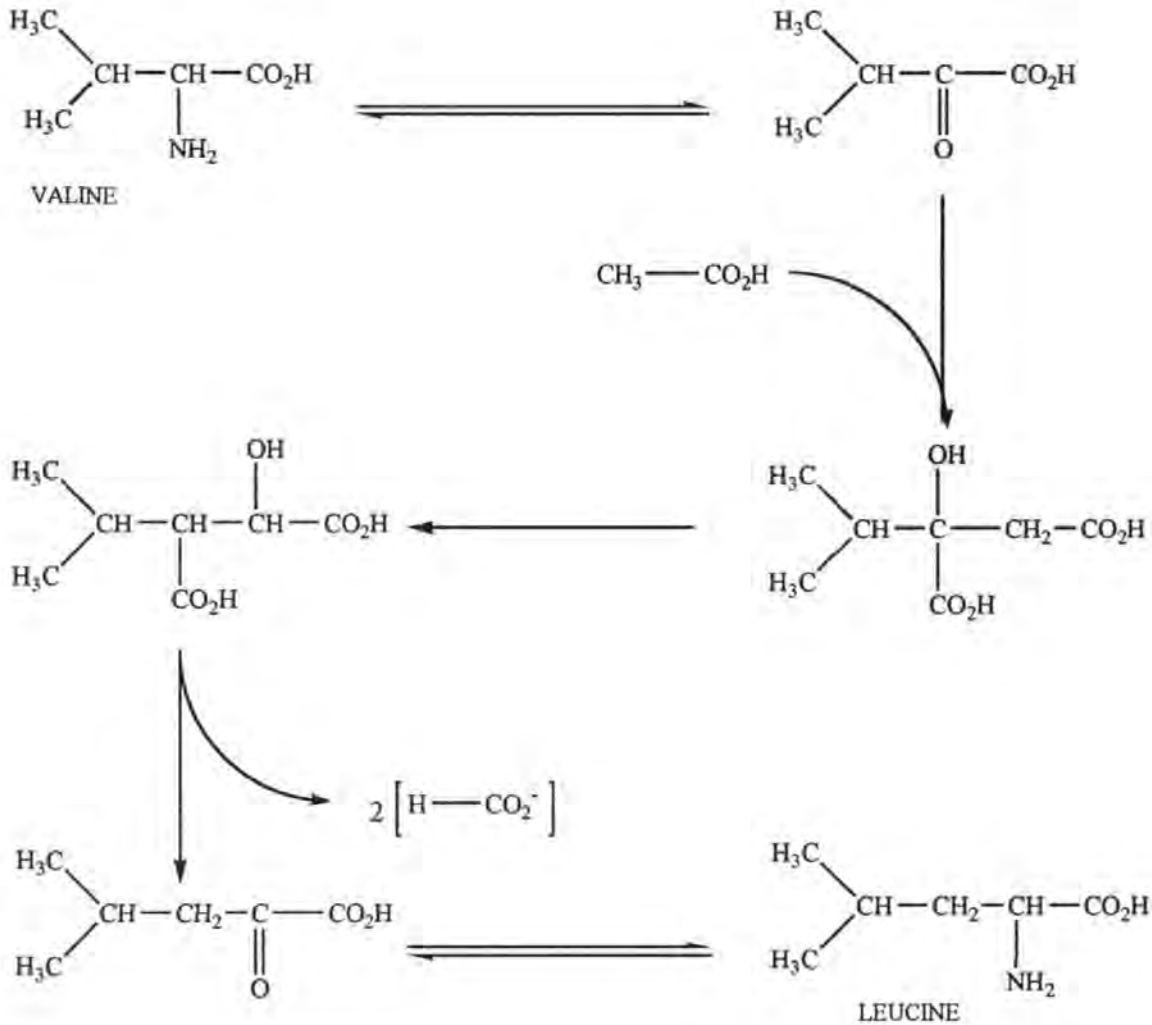


FIGURE 12 CHAIN EXTENSION IN AMINO ACIDS

A number of nitrogenous compounds have been found to act as precursors for glucosinolate biosynthesis and are considered to form part of the biosynthetic pathway between amino acids and glucosinolates. The first demonstration of a nitrogenous intermediate was reported by Tapper and Butler (1967) and Underhill (1967) who found that phenyl[^{14}C]acetaldoxime was a more efficient precursor of benzylglucosinolate than [^{14}C]phenylalanine. Phenylacetaldoxime was found to be a naturally occurring aldoxime in *Tropæolum majus* L. formed from phenylalanine. The extent of incorporation of ^{14}C from [^{14}C]isobutylaldoxime and *3*-phenyl[*2*-^{14}C]propionaldoxime into isopropylglucosinolate (glucocochlearin) and *2*-phenylethylglucosinolate (gluconasturtiin) was equal to or greater

than the incorporation of their corresponding amino acids (Tapper and Butler, 1967; Underhill, 1967).

Studies on the biosynthesis of hydroxamic acids in plants have concentrated on *N*-hydroxyamino acids being intermediates in plant metabolism. Kindl and Underhill (1968) fed *N*-hydroxyphenyl[2-^{14}C]alanine to *Tropæolum majus* L. in an attempt to find intermediates between phenylalanine and phenylacetaldoxime. They found the efficiency of conversion of ^{14}C into the aglucone moiety of the benzylglucosinolate to be higher than that from labelled phenylalanine and comparable to that from labelled phenylacetaldoxime (Fig.13).

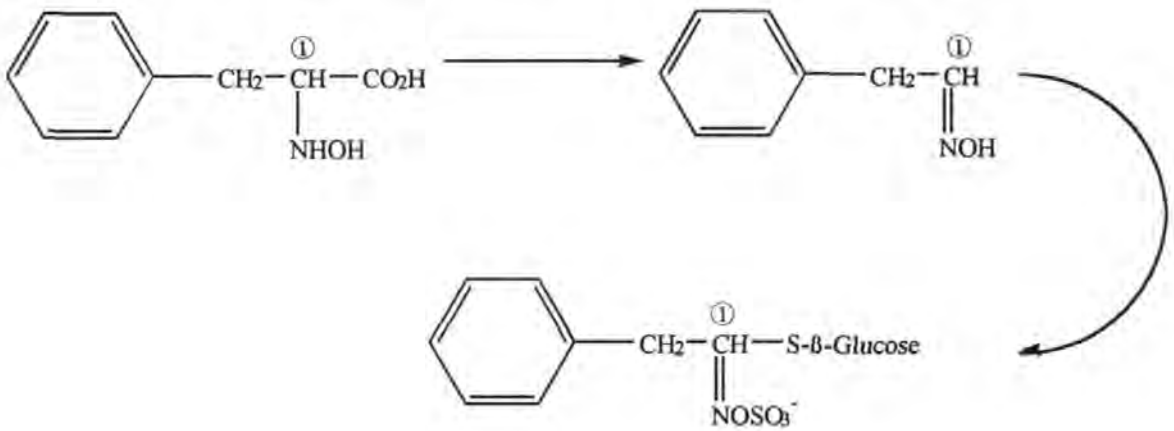


FIGURE 13 CONVERSION OF LABELLED *N*-HYDROXYPHENYLALANINE TO BENZYLGLUCOSINOLATE

They were also able to show the formation of phenylacetaldoxime from *N*-hydroxyphenylalanine using cell-free preparations from *Sinapis alba* L. and *Tropæolum majus* L. It was therefore concluded, though by no means certain, that *N*-hydroxyamino acids are an intermediate between amino acids and aldoximes.

The biosynthetic pathway of glucosinolates shares many features with that of another important group of secondary plant metabolites, cyanogenic glycosides. Cyanogenic glycosides liberate hydrogen cyanide on hydrolysis (Section 1.5.3) the toxicity of which leads to many cases of livestock poisoning and human deaths each year (Harborne, 1988). The glycosides not only act as effective feeding deterrents but have been shown to be

powerful allelopathic agents and it is certain that they play a vital part in enhancing the competitiveness of those plants which produce them (Mann, 1987). Like glucosinolates the biosynthesis of cyanogenic glycosides (Fig. 14) proceed from an amino acid *via* the *N*-hydroxyamino acid to an aldoxime. The enzymology of the biosynthetic pathway of cyanogenic glycosides is reasonably well understood and this knowledge has been incorporated into studies of enzymes involved in glucosinolate production.

Microsomal studies by Halkier and Moller (1991) showed that in the biosynthesis of the cyanogenic glycoside dhurrin in *Sorghum bicolor* L. (Dhurrin) a cytochrome P450 monooxygenase is responsible for the conversion of the amino acid to the aldoxime. Similar work by Koch and co-workers (1992) showed that cytochrome P450 monooxygenases were also responsible for the production of aldoximes in *Manihot esculent* Crantz (Cassava).

Du and Halkier (1996) have shown that in *Tropæolum majus* L. the enzyme responsible for the conversion of phenylalanine to the corresponding aldoxime is sensitive to cytochrome P450 inhibitors, indicating that a cytochrome P450 is involved in the biosynthetic pathway corroborating work by Du and co-workers (1995) that a cytochrome P450 monooxygenase is involved in the conversion of tyrosine to *p*-hydroxyphenylacetaldoxime in *Sinipis alba*.

Microsomal enzyme systems from leaves of *Brassica napus* L. (Rapeseed) have been shown to be unaffected by carbon monoxide or other cytochrome P450 inhibitors. Bennett *et al.* (1993) suggested that the conversion to the aldoxime consists of two separate enzyme catalysed stages. The first step is the *N*-hydroxylation of the amino acid by a NADPH dependent flavin-containing monooxygenase comparable to those found in

mammalian tissue. The second stage is the conversion to the aldoxime which is catalysed by a non-NADPH dependant enzyme, the exact nature of which is still unknown.

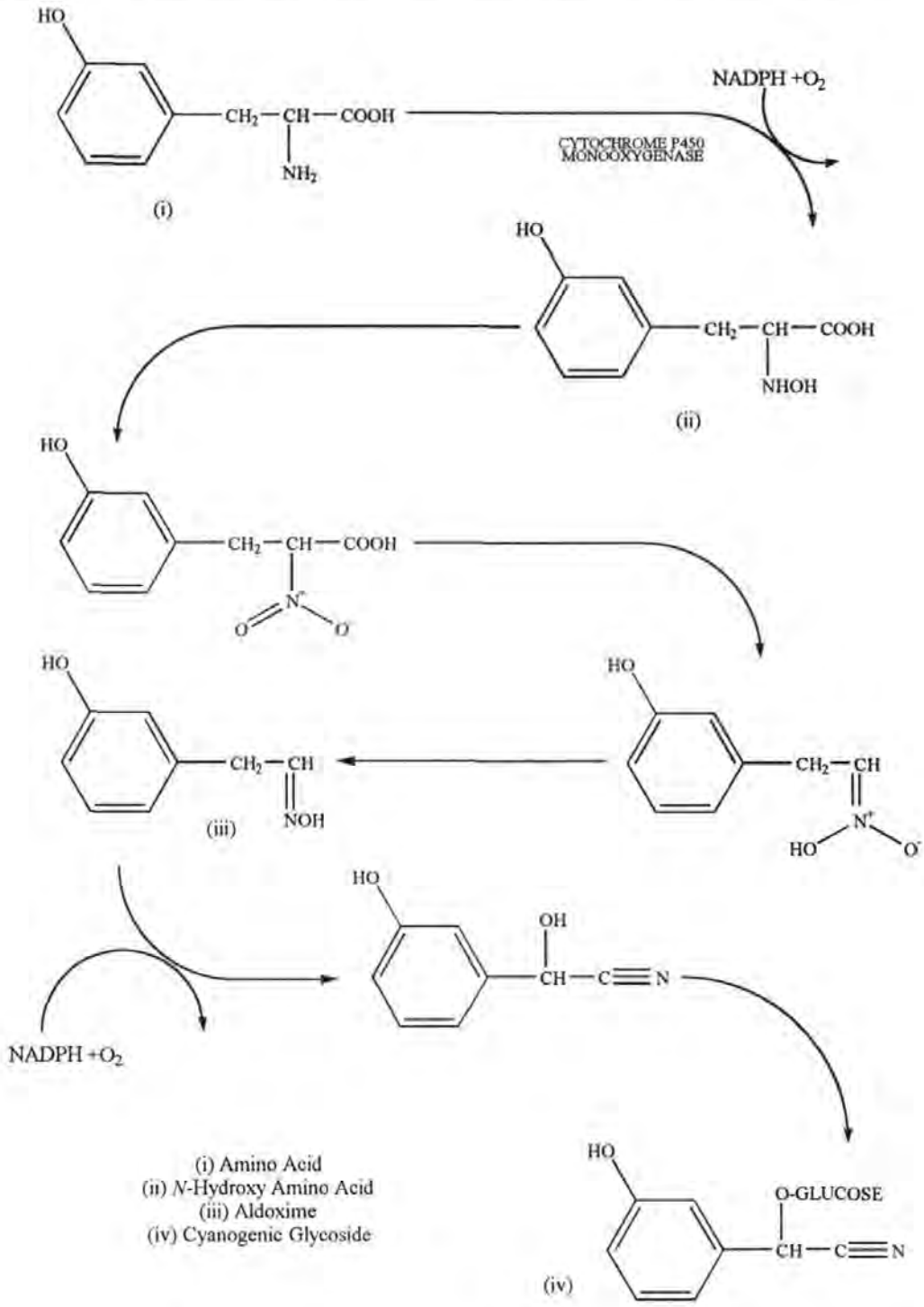


FIGURE 14 BIOSYNTHESIS OF DHURRIN, A CYANOGENIC GLYCOSIDE

A third aldoxime-forming enzyme system has been found in *Brassica pekinenis* (Lour.) Rupr. (Chinese Cabbage). This is a membrane-bound peroxidase that converts

tryptophan to indole-*3*-acetaldoxime (Ludwig-Muller and Hilgenberg, 1988; Ludwig-Muller *et al.*, 1990). Such activity is not restricted to glucosinolate-accumulating plants and indole-*3*-acetaldoxime has been proposed as a precursor of indole acetic acid a plant growth regulator (Helmlinger *et al.*, 1987). Uniquely *Carica papaya* L. (Papaya) contains both classes of secondary metabolite, glucosinolates and cyanogenic glucosides (Spencer and Seigler, 1984). The biosynthetic mechanisms of each have yet to be investigated.

All of the known glucosinolates contain at least two and in some cases three sulphur atoms. The distribution of radioactivity in the glucosinolate sulphur atoms derived from ^{35}S labelled amino acids was in sharp contrast to the distribution observed when inorganic sulphur compounds were fed to *Brassica oleracea*. Approximately 80% of the sulphur incorporated into prop-*2*-enylglucosinolate from [^{35}S]methionine and, more effectively, [^{35}S]cysteine was located into the thiohydroximate sulphur, whereas 89% of the sulphur from sodium [^{35}S]sulphate was incorporated into the sulphate sulphur of the glucosinolate (Wetter, 1964). Although it is now known that cysteine is the sulphur donor the enzyme catalysing the sulphur-donation has not yet been characterised.

Data confirming the precursor role of thiohydroximates in the biosynthesis of glucosinolates were reported by Underhill and Wetter (1969) who showed that there were high levels of conversion from phenyl[*2*-^{14}C]aceto[^{35}S]thiohydroximate and *S*-(*β*-D-glucopyranosyl)phenylacetothiohydroximic acid (desulphobenzylglucosinolate) to benzylglucosinolate (Fig. 15). These results have been confirmed and extended by the demonstration that phenylacetothiohydroximate occurs naturally in *Tropæolum majus* L. (Matsuo and Underhill, 1969). The soluble enzyme UDPG-thiohydroximate glucotransferase has been isolated from a number of glucosinolate containing plants (Matsuo *et al.*, 1971) and shown to catalyse the glucosylation of the thiohydroximate.

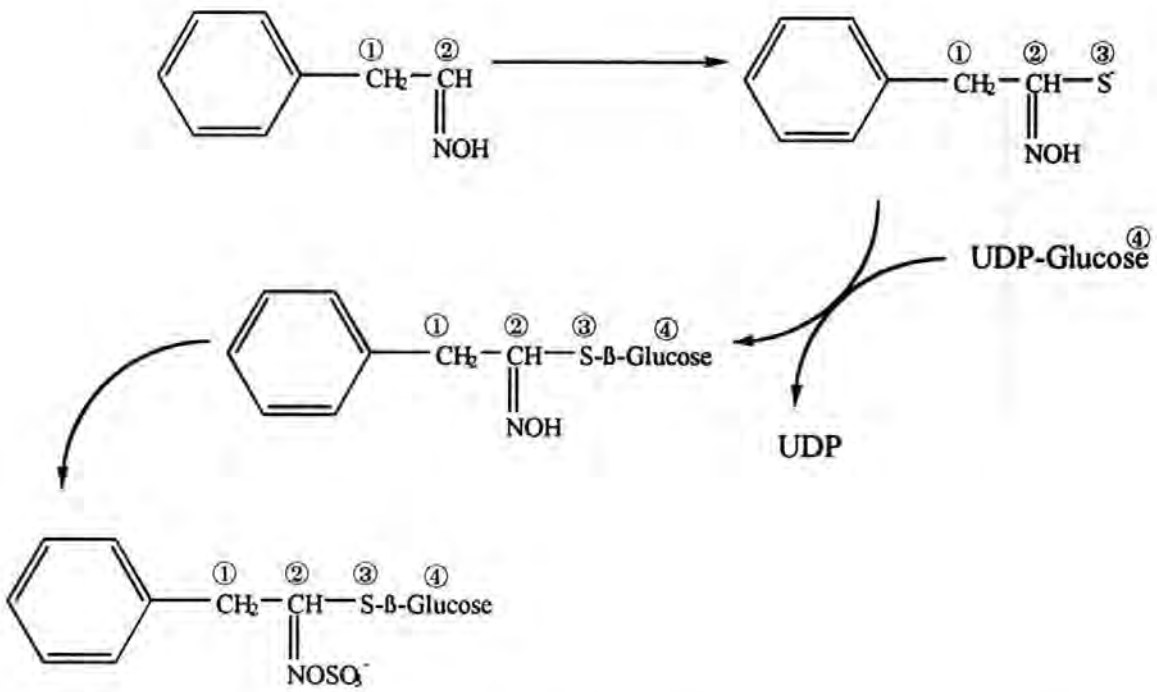


FIGURE 15 **CONVERSION OF LABELLED PHENYLACETOTHIOHYDROXIMATE TO BENZYLGLUCOSINOLATE**

The enzyme desulphoglucosinolate sulphotransferase has also been shown to be present in plants which contain glucosinolates and is thought to be responsible for the conversion from desulphoglucosinolate to the glucosinolate along with *3'*-phosphoadenosine *5'*-phosphosulphate which acts as the sulphate donor. The two enzymes have been purified and shown to be non-specific with respect to the nature of the side chain (Guo and Poulton, 1994).

Further side chain modification may occur during glucosinolate biosynthesis. Several glucosinolates are known whose aglucones contain phenolic and/or aliphatic hydroxyl groups and without exception their deoxy analogues are known. Underhill and Kirkland (1972) fed *2*-amino-*4*-phenyl[*2*-^{14}C] butyric acid to *Reseda luteola* and from the specific radioactivities of the glucosinolates isolated, they concluded that *2*-hydroxy-*2*-phenylethylglucosinolate was formed from *2*-phenylethylglucosinolate. *2*-[^{14}C]Phenyl ethylglucosinolate was also fed to the plants and converted into the hydroxylated

glucosinolate. These results strongly suggest that such hydroxylation is the last step of the biosynthesis sequence. Work by Kindl (1965) suggests that the introduction of phenolic hydroxyl groups occurs at the amino acid stage of biosynthesis.

Studies on the biosynthesis of prop-*2*-enylglucosinolate have led to the suggestion that it may be derived from *3*-methylthiobutylglucosinolate by elimination of the methylthio group. A similar suggestion was made by Ettlinger and Kjær (1968) who noted that but-*3*-enyl-, *2*-hydroxybut-*3*-enyl-, *4*-methylthiobutenyl- and *4*-methylsulphinylbutyl-glucosinolates (Fig. 16) co-exist in a number of *Brassica* species, and that similar glucosinolate combinations appear in *Allysum* and *Iberis* species.

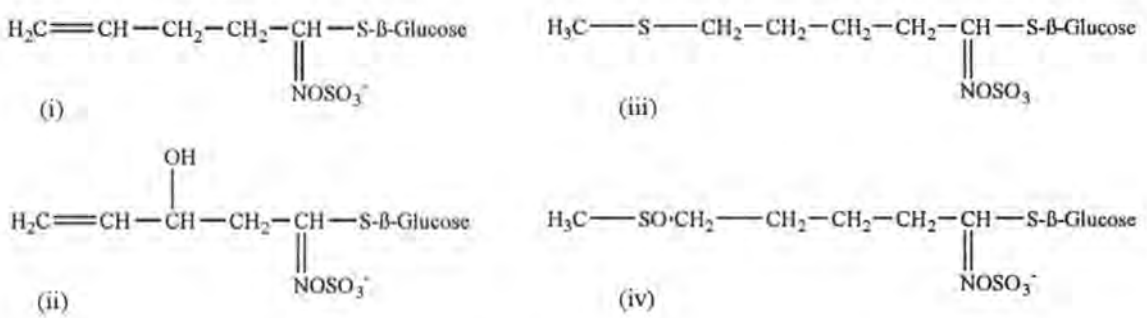


FIGURE 16 STRUCTURES OF (i) BUT-*3*-ENYL, (ii) *2*-HYDROXYBUT-*3*-ENYL, (iii) *4*-METHYLTHIOBUTYL, (iv) *4*-METHYLSULPHINYLBUTYL GLUCOSINOLATES

Distributional data such as these, combined with the results of biosynthetic studies on prop-*2*-enylglucosinolate and its higher homologues strongly indicate that the terminal double bond in a straight-chain glucosinolate probably arises from the elimination of methanethiol or oxidised equivalent (Underhill *et al.*, 1973).

The biosynthesis of prop-*2*-enylglucosinolate is used as an example of a glucosinolate biosynthestic pathway (Fig. 17).

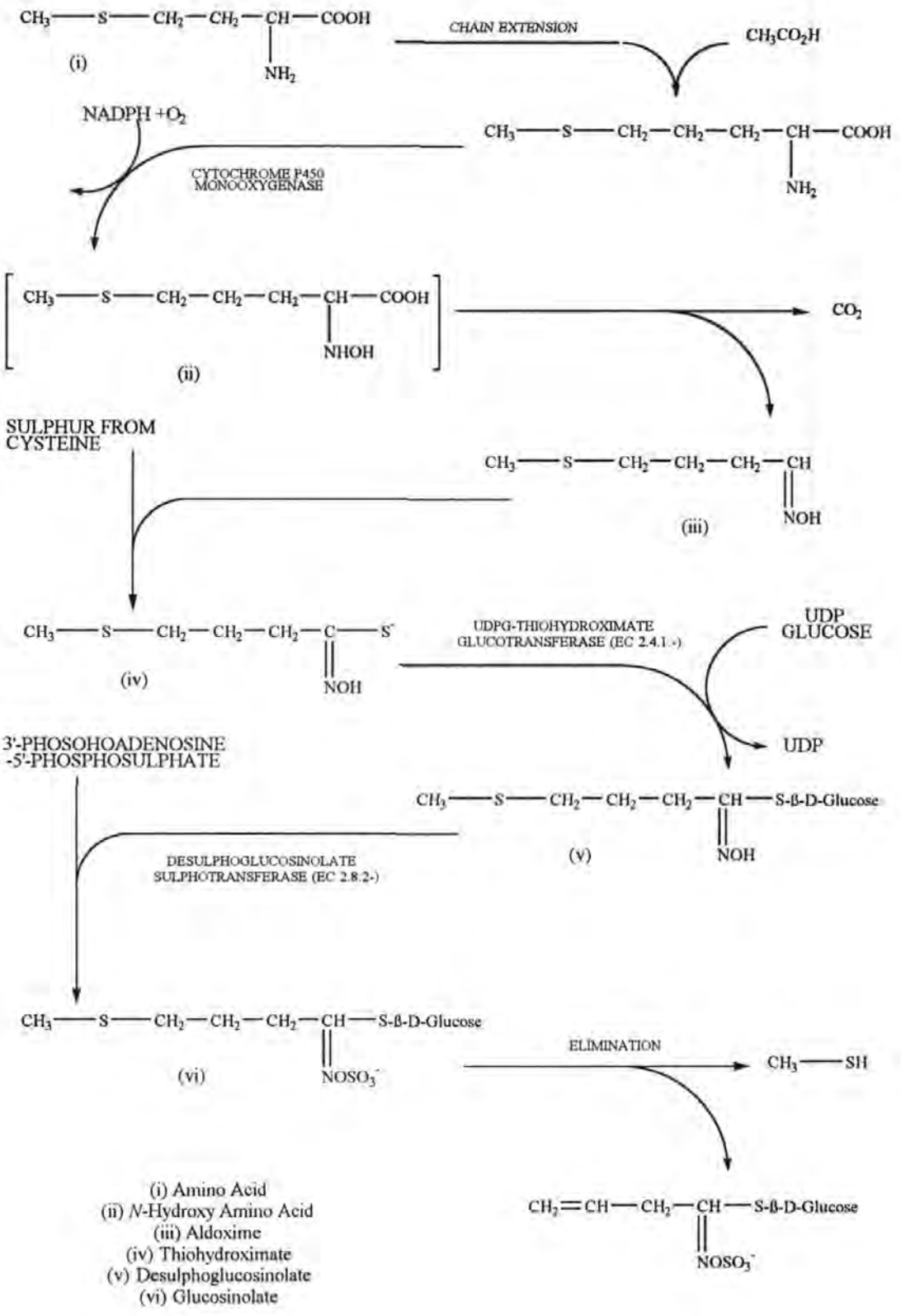


FIGURE 17 BIOSYNTHESIS PATHWAY FOR PROP-2-ENYLGLUCOSINOLATE

1.5.3 Myrosinase Degradation of Glucosinolates

An important feature of glucosinolate chemistry is the enzymatic hydrolysis of glucosinolates under the action of the thioglucosidase enzyme myrosinase (EC 3.2.3.1). The myrosinase-glucosinolate system has long been the defining phytochemical character of the order Capparales (Hegnaur, 1986). Enzymes with myrosinase activity have been found in certain fungi (Reese *et al.*, 1958), in intestinal bacteria (Oginsky *et al.*, 1965) and in mammalian tissues (Goodman *et al.*, 1959). Myrosinase degradation of glucosinolates produces compounds that effect the value of glucosinolate-containing plants when used as food for humans or for feeding animals (Chew, 1988). In the intact plant, enzyme and substrate are prevented from coming together, presumably as an adaptive measure to avoid auto-toxicity, but following cell disruption enzyme and substrate come into contact. The resulting hydrolysis involves the cleavage of the thioglucoside bond and yields free glucose and an aglucone intermediate which undergoes spontaneous degradation to one of a number of toxic metabolites (Fig. 18).

The degradation of glucosinolates is comparable to the hydrolysis of cyanogenic glycosides to produce HCN. Cyanogenesis occurs after tissue disruption by herbivory or fungal attack. In intact plants the HCN is normally stored in the form of cyanohydrins stabilised by *O*-glycosidic linkage to sugar (mainly glucose) residues. The catabolic release of HCN from these precursors is initiated by the action of a specific β-glucosidase which provides free sugars and unstable cyanohydrins that decompose either spontaneously or by hydroxynitrile lysases to give HCN plus aldehyde or ketone (Fig. 19)

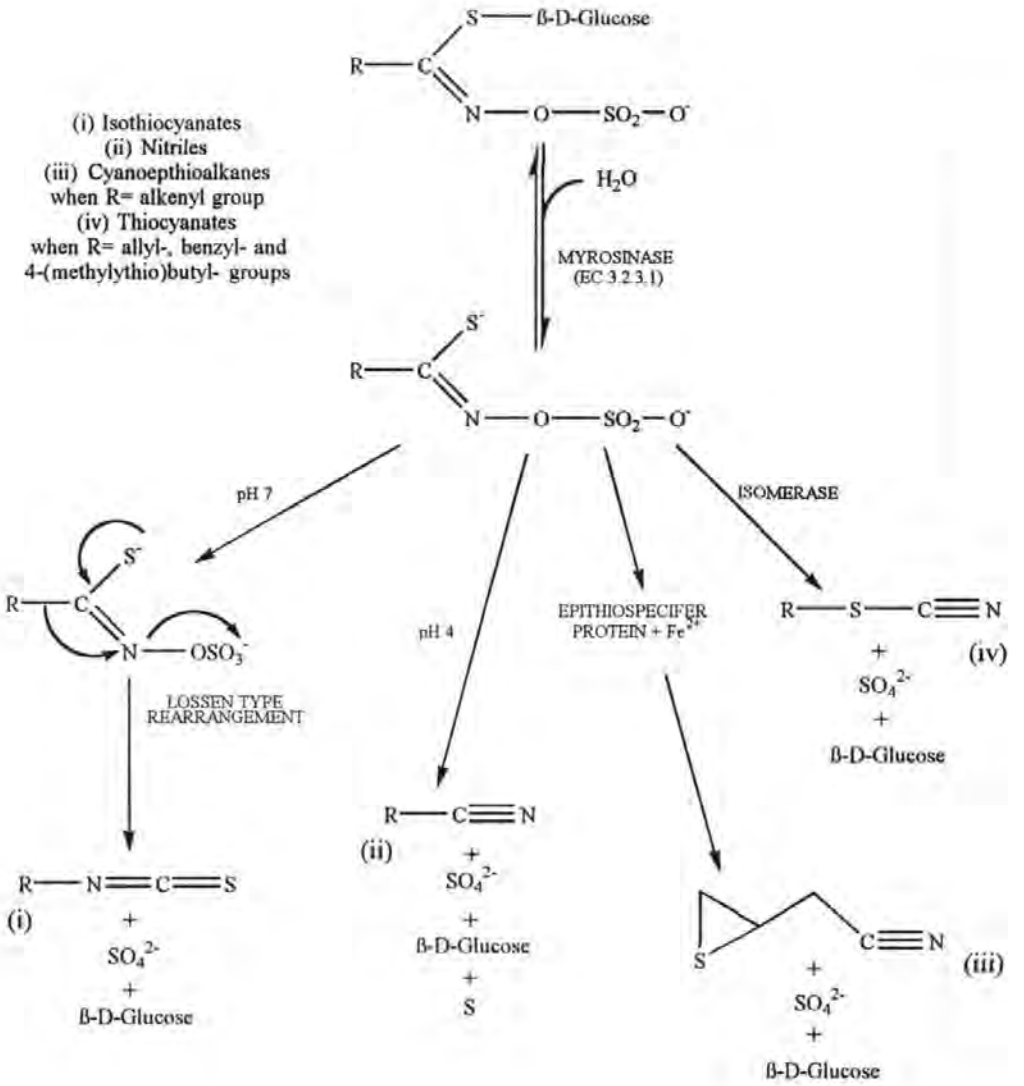


FIGURE 18 **MYROSINASE CATALYSED DEGRADATION PATHWAYS OF GLUCOSINOLATES**

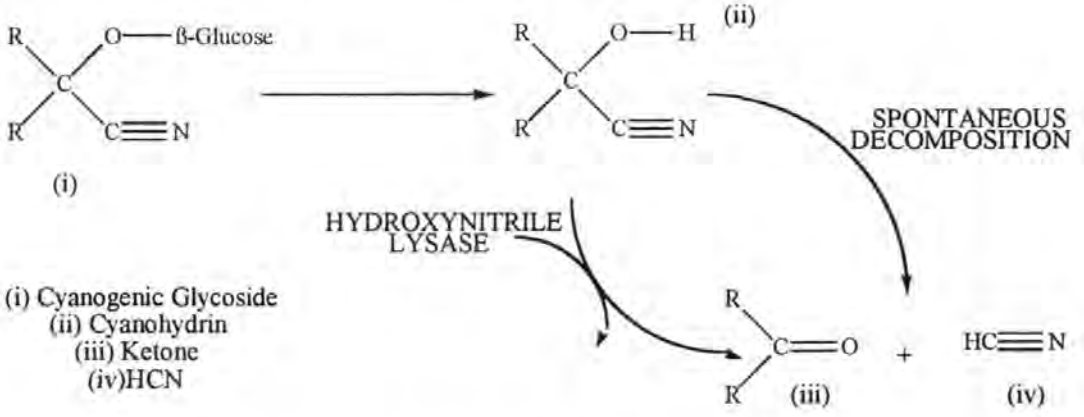


FIGURE 19 **DEGRADATION OF A CYANOGENIC GLYCOSIDE**

The most commonly cited breakdown products of glucosinolates are the isothiocyanates and nitriles but, depending on conditions in the hydrolysis medium and the structure of the parent molecule, a number of other products may arise. A β-OH group on the R-chain of *2*-hydroxy-*3*-butenylglucosinolate (progoitrin) facilitates the cyclisation of the corresponding isothiocyanate to give *S*-*5*-ethenyl-*2*-oxalidinethione (*5*-OZT). Protein co-factors may direct rearrangement of the aglucone to produce cyanoepithioalkanes at the expense of aliphatic nitriles (Cole, 1978). Furthermore isothiocyanates may undergo spontaneous degradation to thiocyanates (Gil and MacLeod, 1980). These thiocyanates can inhibit the uptake of iodine in the thyroid and large amounts are known to have negative effects on the liver. More recently, interest has focused on the potential mutagenic and carcinogenic properties of naturally-occurring isothiocyanates (Ioannou *et al.*, 1984).

The complexity of the myrosinase-glucosinolate system indicates its importance in the life of the cruciferous plants. Glucosinolates may act as a sink for nutrients like nitrogen and sulphur and the hydrolysis products may play important roles in the defence system of the plants against micro-organisms and insects. The number of different glucosinolates and isoenzymes that have been reported may indicate that specific hydrolytic products are required for certain developmental stages or situations. Indoleglucosinolates may, for example, be a sink for the production of the plant hormone indoleacetic acid (IAA) and thereby be involved in growth regulation (Bones and Rossiter, 1996).

Many forms of myrosinase have been shown to exist in a number of plants. MacGibbon and Allison (1970) found that the pattern of isomyrosinase enzymes differs between species within a plant family. It has also been found that there are variations on these distinct patterns depending on whether the extracts analysed were from the root, stem or seed. It has been reported that seeds of *Sinapis alba* L. (White Mustard) contained 14

different myrosinase isoenzymes (Buchwaldt *et al.*, 1986). The degradation rate of glucosinolates increases considerably in the presence of ascorbic acid (Grob and Matile, 1980) and the degree of activation by ascorbic acid has been shown to be different for each of the isoenzymes (James and Rossiter, 1991). The ascorbic acid does not participate in the myrosinase-glucosinolate reaction nor is it involved in the association of the enzyme units. The activation seems to result from a conformation change in the protein structure which in turn leads to an enhanced reaction rate when the effector binding sites are filled (Ohtsuru and Hata, 1973). High concentrations of ascorbic acid inhibit and low concentrations activate the myrosinase (Björkman, 1976). Optimal ascorbic acid concentrations for myrosinase have been reported to be between 1.8 and 2.0 mmolar (Ohtsuru and Hata, 1979; Grob and Matile, 1980).

It is thought that the ascorbic acid, glucosinolates and myrosinase exist together in a myrosin grain/vacuole of a myrosin cell (Fig. 20) where the ascorbic acid concentration is high enough to suppress the enzyme. Disruption of the tonoplast membranes by, for example, attack by a pest insect would allow the vacuole's contents to spill out. There would then be a reduction in ascorbic acid concentration due to dilution which would activate the enzyme system.

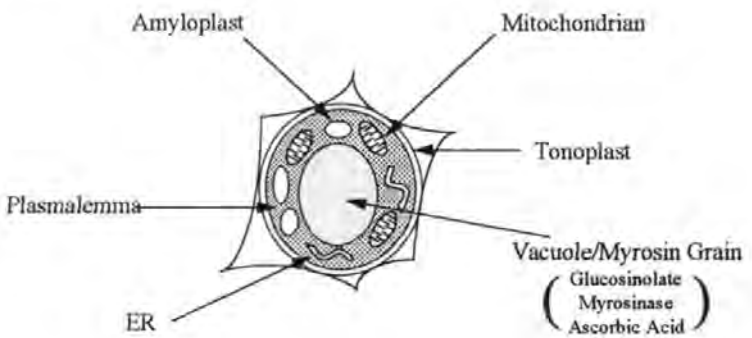


FIGURE 20 SCHEMATIC MODEL OF A MYROSIN CELL (LÜTHY AND MATILE, 1984)

A small protein (30-40 kDa) called epithiospecifier protein (ESP) has been isolated from *Crambe abyssinica* (Crambe) seeds (Tookey, 1973) which, although it has no myrosinase activity itself, interacts with myrosinase to promote the transfer of sulphur from the S-glucose moiety to the terminal alkenyl moiety. Degradation of *2*-hydroxy-*3*-butenylglucosinolate in the absence of ESP produces mainly oxazolidine-*2*-thione, whereas in the presence of ESP the reaction produces mainly epithionitrile. ESP is unique in that it specifies the reaction product not the substrate although its function *in vitro* is unknown.

1.5.3.1 Isothiocyanates

The most common enzymatic hydrolysis products of glucosinolates under neutral and high pH levels are isothiocyanates. Some isothiocyanates are valued for their pungent biting taste such as allylisothiocyanate (derived from prop-*2*-enylglucosinolate) in mustard. All compounds in this family are lachrymators and irritants. Isothiocyanates are similar to isocyanates except that they have a lower reactivity due to the difference in electronegativity of the sulphur and oxygen respectively. This also leads to the central carbon atom of the isothiocyanate having less electrophilic character. Attack by nucleophilic species can occur and hence nucleophilic additions constitute the majority of isothiocyanates. These volatile liquids are also immiscible with water

1.5.3.2 Nitriles

There is mounting evidence that nitriles are the predominant hydrolysis products in natural conditions. For example nitriles have been found to predominate in the digestive tract of poultry following rapeseed ingestion (Smith and Cambell, 1976) and autolysis of plant tissue appears to favour nitrile release (Cole, 1976). Their production is favoured at acidic pH levels where there is liberation of elemental sulphur without a change to the

carbon structure of the aglucone (Kjær, 1960). *In vitro* studies have shown that the presence of ferrous ions in the hydrolysis medium also directs the breakdown of glucosinolates to nitriles (Tookey and Wolff, 1970). Thiol compounds, such as glutathione, enhance the action of Fe^{2+} in favouring nitrile production (Searle *et al.*, 1983).

Nitriles certainly appear to be among the most toxic of the glucosinolate breakdown products, but the actual biochemical nature of this toxicity is still unclear. Generally the toxicity of nitriles is attributed to their effects on the liver and kidneys with increased organ weights (Cole, 1976).

1.5.3.3 Thiocyanates

Isothiocyanates are isomeric with thiocyanates which are a less common breakdown product of glucosinolates. Their production is favoured by low temperature and similar pH conditions as isothiocyanates. Of the naturally occurring glucosinolates only three have been observed to undergo enzymatic degradation to the thiocyanate. These are prop-*2*-enylglucosinolate, benzylglucosinolate and *4*-(methylthio)butylglucosinolate. Their production is thought to involve an isomerase enzyme causing *Z-E* isomerisation of the aglucone. The Lossen type rearrangement to the isothiocyanates is blocked so the R side chain must migrate to the sulphur atom via a stable carbocation. The *2*-propenyl- and benzyl- carbocations are stabilised through a number of resonance structures through which the thiocyanate can be formed. The stabilisation mechanism in *4*-(methylthio) butylglucosinolate is unclear (Hasapis and MacLeod, 1982).

1.5.3.4 Oxazolidine-2-thiones

These cyclic compounds are formed by the spontaneous rearrangement of *2*- and *3*-hydroxy substituted isothiocyanates on autolysis of the parent glucosinolate (Fig. 21). One

such oxazolidinethione is S-*5*-ethenyl-*2*-oxazolidinethione (5-OZT or goitrin) of which the goitrogenic effects are well documented.

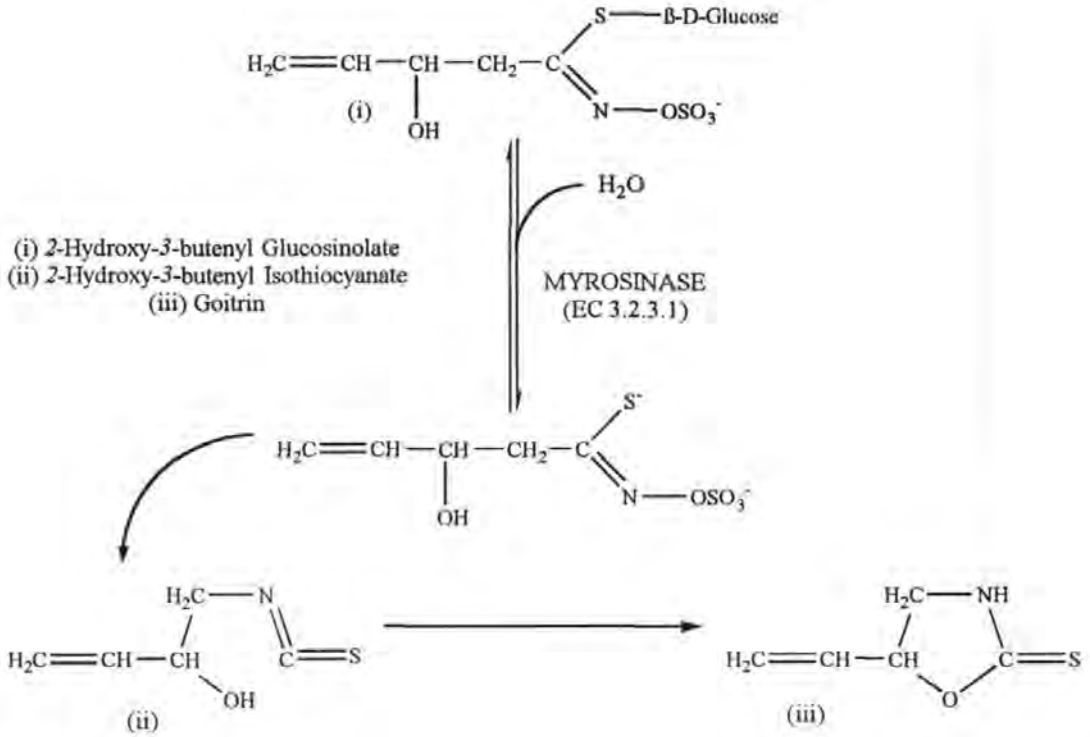


FIGURE 21 DEGRADATION OF *2*-HYDROXY-*3*-BUTENYLGLUCOSINOLATE TO *S*-*5*-ETHENYL-*2*-OXAZOLIDINETHIONE

The goitrogenic effects appear to be mediated by interference with the organic iodination of thyroxine during the biosynthesis the of thyroid hormone. Although this mechanism of toxicity distinguishes 5-OZT from the other brassica derived goitrogens (isothiocyanates and thiocyanates) the manifestations are the same with increased thyroidal weight being a consistent feature (Fenwick *et al.*, 1983).

1.5.3.5 1-Cyanoepithioalkanes

Cyanoepithioalkanes are produced from the hydrolysis of alkenyl glucosinolates when myrosinase co-occurs with a small labile epithiospecifier protein (Tookey, 1973) and involves the migration of sulphur across the double bond of the aglucone.

1-Cyano-*3,4*-epithio derivatives of butenyl- and pentenylglucosinolates have been isolated from a number of cruciferous species (Cole, 1976).

1.5.4 Chemical Synthesis of Glucosinolates

There are two well documented main synthetic pathways which produce glucosinolates. One proceeds via a thiohydroximate intermediate (Kjær, 1952; Ettlinger and Lundeen, 1957; Walter and Schaummann, 1971). It involves the conversion of a Grignard product into the dithioacetate which is then reacted with hydroxylamine to give the thiohydroximate. The addition of acetobromoglucose gives a fully acetylated desulphoglucosinolate. Sulphation using pyridine:sulphur trioxide is followed by the removal of the protecting acetyl groups by methanol and ammonia.

The second pathway (Kjær and Jensen, 1968; Viaud *et al.*, 1992) involves the nitration of an appropriate bromo compound which in turn is converted to the stable sodium nitronate salt. The chlorooxime is achieved by a reaction with either lithium chloride or thionyl chloride and the addition of a fully acetylated thioglucose gives rise to the acetylated desulphoglucosinolate which is sulphated and deprotected as described above. Glucosinolates are usually isolated as potassium salts by the addition of potassium hydrogen carbonate at the sulphation stage.

A more detailed discussion of the chemistry involved in the production of glucosinolates is given in Chapter 4.

1.6 AIMS OF WORK

Contact chemoreception plays a decisive part in the oviposition behaviour of *Delia radicum* but although the glucosinolates are implicated the evidence for their importance is incomplete. Traynier (1967) first showed that prop-*2*-enylglucosinolate (sinigrin) stimulated

oviposition though it was a weaker stimulant than natural plant extracts. Electrophysiological studies (Roessingh *et al.*, 1992) have shown that tarsal contact chemoreceptors occur in female cabbage root fly that are sensitive to glucosinolates. The biological activity of a range of glucosinolates was investigated using electrophysiological recording and a bioassay method based on the use of surrogate leaves coated with test compounds. A significant correlation was found between overall length of the side chain of the glucosinolate molecule and biological activity but no other structure-activity work has been carried out. The most stimulating fraction of plant surface extract contained no glucosinolates and it was concluded that compounds in addition to glucosinolates play an important role in oviposition.

The main aim of this work was to synthesise various glucosinolates and related compounds and investigate the structure-activity relationship between the compounds and their efficacy as oviposition stimulants for the cabbage root fly. This would, hopefully, lead to the identification of which part or parts of the glucosinolate structure are primarily responsible for oviposition stimulation. An ultimate aim would be to identify a powerful oviposition stimulant which could then possibly be used either in conjunction with the commercial monitoring trap, Brassiceye™ or in some other way to provide greater crop protection and minimise pesticide use.

To achieve this goal it was first necessary to establish a sound bioassay procedure thereby enabling the accurate and quantifiable assessment of the various compounds as oviposition stimulants.

2. BIOASSAY

2.1 BACKGROUND

2.1.1 General Procedures

In its widest sense, the term "bioassay" covers all experiments where the potency of a chemical or physical cue is measured by reference to a standardised insect colony (Busvine, 1971). To make progress in experimental work, it is obviously necessary to be able to obtain consistent results. Bioassays involve two main components, the insects and the test agents; both of which must be standardised, as far as possible, in order to obtain repeatable results.

Many aspects of insect physiology are influenced by environmental factors and the conditions under which an insect has been reared, or maintained for a considerable time before testing, may effect its behaviour (Busvine, 1971). By using insects from a laboratory insect culture it is possible to control and standardise conditions, such as temperature, humidity and photoperiod, under which the insects are both reared and tested. In addition it is possible to maintain the population size of a culture at a certain level and therefore control the size of the insects as this will also effect some aspects of their behaviour. Experiments with mosquitos *Anopheles quadrimaculatus* have shown that undersized adults reared from overcrowded colonies not only have lower biting rates but are less consistent (Terzian, 1949).

Field testing is financially costly and time consuming and uncontrolled variables make repeatability difficult to achieve. The use of a laboratory test arena is both cheap and

convenient and allows standardisation of the bioassay for example, in the bioassay of chemical test agents dosage can be measured accurately.

Standardised laboratory conditions may differ from field conditions in a number of respects. Cultivated flies may also differ from wild types in some traits. In a behavioural bioassay it is possible that laboratory conditions and the use of cultivated flies introduces artefacts. One such apparent artefact is the positive phototaxis shown by insects, including *Delia radicum* (Hawkes and Coaker, 1979). This behaviour has been interpreted by Hawkes and Coaker (1979) as a part of the escape behaviour as a result of being held in a confined space, such as a culture cage or test arena. In wind tunnel experiments the escape behaviour does not override the response to oviposition stimuli. The oviposition behaviour exhibited by the cabbage root fly under laboratory conditions and observed by Städler and Schöni (1990) closely matches the behaviour observed by De Wilde (1947) in the field.

Uniform illumination of a test arena will reduce the degree of positive phototaxic behaviour exhibited by the insects and the use of test standards can indicate any bias to certain areas of the arena that may exist.

2.1.2 Cabbage Root Fly

Traynier (1967) was the first to make a study of physical and chemical aspects of oviposition stimulation in the cabbage root fly. Experiments were carried out in a glasshouse with no regulation of temperature or humidity. The temperature was reported to fluctuate between 10 - 32 °C and a tray of damp peat on the floor of the cage kept the relative humidity anywhere above 50 %. A range of concentrations of plant juices and aqueous solutions of chemicals were tested as oviposition stimulants in test-units of concentric glass tubes embedded in sand (Fig. 22).

In each experiment the test-units were placed in random positions 15 cm apart in a cage containing 25 flies of each sex. The flies were 5 or more days old, the experiment was replicated each day and was repeated on 3-9 successive days with the same cages of flies.

A lack of standardisation of a number of factors may question the accuracy of Traynier's results. In addition to the behaviour of the insects, the release rates of the isothiocyanates, carbon disulphide and the other volatile compounds tested would be affected by changes in the environmental conditions as would the production of such compounds by the hydrolysis of the plant juices. A change in the number of eggs laid during 'replicate' experiments could be a result of a change in the landing rate of the insect. This is, in turn, related to a change in the release rate of volatile compounds from the test site. Despite the fact that the number of eggs laid by a female fly changes with age and conditions (Smithers, Pers. Comm.) there was no standardisation of the age of the adult flies used in the bioassays and the same set of flies were used more than once.

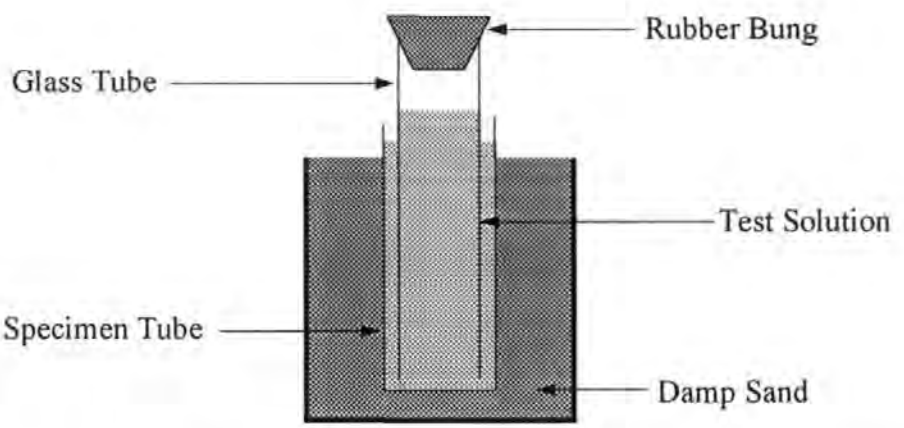


FIGURE 22 SAGITTAL SECTION OF A TEST-UNIT FOR OVIPOSITION STIMULI (TRAYNIER, 1967)

The approach adopted by Nair and McEwen (1976) addressed some of these standardisation problems. Their experiments were conducted in controlled environment chambers at a constant temperature of 20 °C, 70% relative humidity and 16 hour

photoperiod. However, the number of flies present could only be estimated as being approximately 50 to 75 pairs. Dead flies were replaced daily by newly emerged insects so there would be groups of flies all differing in age and sexual development.

A gelatin-alphacel block was developed by Nair and McEwen as a suitable medium into which various substances could be incorporated and tested for oviposition - inducing activity. It was assumed that any test compound would be uniformly distributed throughout the block and, although, it was acknowledged that the concentration of test materials incorporated into the gelatin-alphacel block increased progressively as the block lost water due to evaporation, no investigation into the degree of change was made. The techniques used would appear to have several limitations in a quantitative study of oviposition stimulation.

A number of workers have since investigated the effect of foliar form, colour and surface characteristics on oviposition behaviour by the cabbage root fly each performing their experiments under controlled laboratory conditions with standardised test arenas and randomised arrangements of test sites. Roessingh and Städler (1990) did not standardise the numbers of flies used and the ages varied from one to seven days old. Bioassays were also performed over a two week period with the same set of flies. The number and age of the flies used by Kostál (1991, 1993a, 1993b, 1994) were both known though again the same flies were used over a period of time. The standardisation of the number of flies used is important when considering the possiblity of oviposition deterring compounds being present on the eggs (Braven *et al.*, unpublished). The potency of this deterrence will depend on the number of eggs laid on one site whch in turn will be related to the number and age of insects used.

Work assessing the effect of trap background on cabbage root fly landing and capture by Finch (1995) showed that the optimum trap backgrounds for maximum capture of male flies should be grass whereas for females it should be bare soil. His bioassays involved a known number of flies which had all emerged within the same 24 hour period and so were all of a known age. The flies were bioassayed only the once in each experiment.

Städler and Roessingh (1990) and Kostál (1993b) found that the size and shape of the surrogate leaf made little or no statistical difference to the number of eggs laid on or around the model, though it was observed that the female flies did seem to prefer a round or circular model to a square or rectangular one. The absence of a "stem", however, did produce a significant drop in the degree of oviposition that took place, the optimum length being between 4 and 9 cm. Kostál (1991) found that light blue or white oviposition sites were most preferred by gravid females.

Roessingh and Städler (1990) had found that a uniform paraffin wax layer did increase the number of eggs laid on a model though it was not thought to play any chemosensory role. They applied paraffin wax to a number of leaves in various ways to give each a different surface texture. When the level of oviposition on each was studied it was found that a leaf with a smooth uniform layer of wax would have eggs laid on or round it. Leaves with an uneven or patterned surface would have a greatly reduced number of eggs compared with an uncoated leaf.

To ensure the behavioural bioassays in this study were sound and reliable, a number of factors were to be standardised. The female flies used would be from a long established laboratory culture. The numbers of flies used for each experiment would be kept constant throughout and would be such that even at maximum egg deposition by all the insects oviposition detterence effects would be minimal. The flies would all be of the same age and

would only be used once. The sites were to be white circular surrogate leaves with a stem although without a wax coating to allow a more even distibution of the test solutions over the model's surface. A standard amount of solution would be applied to each model leaf with a check of the compound's stability on a leaf to ensure that there was no decompositon over the period of the bioassay. A random arrangement of test, standards and blank sites equidistant from each other were to be presented to the adult females over the period of 24 hours under carefully controlled environmental conditions.

2.2 CABBAGE ROOT FLY CULTURE

Flies were collected from the National Vegetable Research Station Wellesborne to start a Wellesborne culture. At the same time pupae were collected from the Rumleigh Experimental Station in the Tamar Valley to start a Plymouth culture. In 1986 the cultures were merged to provide a general stock culture.

The fly culture was maintained in an environmentally controlled room (18 hours of light:6 hours of darkness: 21±1.5 °C and 70±10 % relative humidity) using a method similar to Finch and Coaker (1969). Pupae ready to emerge were introduced into a cage with water and a 10% sucrose solution in cotton wool within petri dishes. Each week the pupae would be transferred into a new cage so that each cage contained flies that had emerged within a seven day period. Seven days after emerging the flies were given a mixture of sugar and yeast hydrolysate on which to feed in addition to the water and sugar solutions. They were also presented with a swede in fine sand every other day as an oviposition site on which the resulting larvae were reared. After four weeks the flies were terminated.

For the bioassays sufficient numbers of pupae ready to emerge were placed in a separate holding cage for 24 hours. Any flies that emerged were then kept under the same

conditions as the main culture. The remaining pupae were transferred into another holding cage for a further 24 hours and the process repeated until all the pupae had hatched. This ensured that all the flies in each holding cage were of the same age within 24 hours. After five days the flies were given the sugar and yeast hydrolysate and at seven to eight days the flies were used in the bioassays.

2.3 BIOASSAY PROCEDURE

Bioassay experiments were performed under the same environmental conditions as the main culture in screen cages measuring 50 x 50 x 50 cm, on the floor of which were placed nine imitation or surrogate leaves equidistant from each other in a random grid arrangement which would minimise interference between sites and show any bias towards particular areas of the cage (Fig. 23). Three sites were blanks (B), three were control standards (T_1) and three were treated with the compound under test (T_2). Ten female flies of seven days old and between 12 and 15 mg in weight were introduced into the cage for 24 hours. It was found that the numbers of eggs laid by flies below 12 mg in weight were up to 50 % less than the numbers laid by flies over 12 mg. After 24 hours the "leaves" were removed and the contents of each petri dish were emptied into a beaker of water and stirred. The number of eggs that had floated to the surface was then counted.

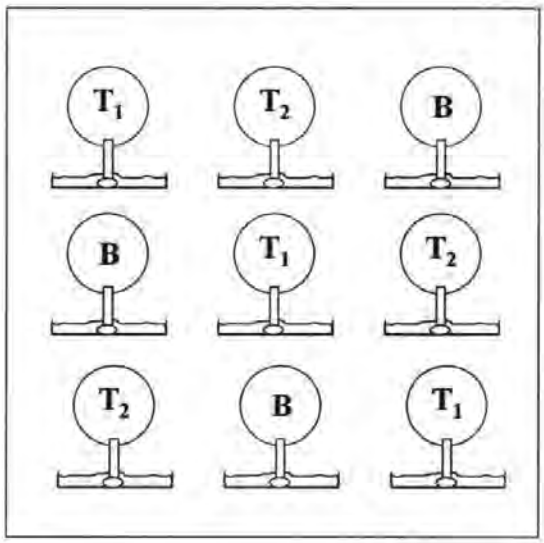


FIGURE 23 LAYOUT OF SURROGATE LEAVES ON THE BIOASSAY CAGE FLOOR

The floor of the test cage was dark in colour, a factor known to aid orientation and the oviposition process (Kostál and Finch, 1994) and uniformly illuminated from above.

The inside of the cage was washed down with warm water after each bioassay and allowed to dry at room temperature.

2.4 SURROGATE LEAF DESIGN

The design of the surrogate leaf structure was adapted from the work completed by Roessingh & Städler (1990) and Kostál (1993) on physical stimuli.

The main leaf body was originally produced by resting a 9 cm in diameter circle of filter paper on a watch glass of similar size so that only the very edge of the paper came into contact with the glass. A quantity of test solution was applied to the paper using a Pasteur pipette. The amount of solution used was just enough to dampen the paper and not to soak it. The stem was made by wrapping a section of polythene tubing 5 cm in length and 0.5 cm in diameter in filter paper which was also dampened with test solution. Both structures were dried under vacuum over calcium chloride. Once dry the leaf was placed into a slot cut into the stem. The leaf model was then fixed into the centre of a petri dish 9 cm in diameter and

approximately 1.5 cm in depth. For each bioassay three leaves were treated with 0.6 ml of aqueous test solution, three were treated with 0.6 ml of 10^{-4} Molar aqueous prop-*2*-enylglucosinolate solution as the control standards and the final three were treated with 0.6 ml of water as the blanks. Finally fine sand was placed into the dish around the leaf in which the flies could lay their eggs.

Early bioassay experiments using varying concentrations of prop-*2*-enylglucosinolate with these leaf models failed to produce any response from the flies. When tested with 1 cm cubes of swede placed in petri dishes filled with sand as the control standards there was still no oviposition around the leaf models but 20 - 40 eggs were laid around each of the swede cubes. This suggested that the flies were healthy and that the procedure was inappropriate and had to be altered. It was decided to include some additional features; one of these was to coat the structure with a layer of wax with a mixture of the test solution and methanol sprayed on to the surface using a chromatography spray.

The new leaf design consisted of a 9 cm diameter circle of white cardboard with vertical folds 1 cm apart which was slotted into a plastic tube stem of 5 cm in length and 0.5 cm in diameter. The folds mimic the veins of a leaf which the flies are known to follow on their way to the stem where they can climb down onto the ground around the plant to lay their eggs. The structure was then coated with a uniform layer of paraffin wax (Merck, m.p. 42-44°C) by a short immersion into warm water with a floating layer (0.6 - 1 cm) of the molten wax on top. Three leaves were sprayed with 0.6 ml of a test solution at a known concentration in 75% aqueous methanol, three were treated with 0.6 ml of 2.5×10^{-5} Molar solution of prop-*2*-enylglucosinolate in 75% aqueous methanol as the standards and the final three were treated with 0.6 ml of aqueous methanol as the blanks. Fine sand was again placed into the dish around the leaf in which the flies could lay their eggs.

After each bioassay the plastic tube "stems", sprays and petri dishes were soaked in a 5% $^{v}/_{v}$ Decon solution overnight and rinsed with Milli-Q water before being allowed to dry at room temperature.

2.5 COMPOUND STABILITY

A method of HPLC analysis (Spinks *et al.*, 1984) was used to ensure that the test glucosinolates and other compounds did not decompose when dried on to the surrogate leaves.

Nine leaves were made up as normal and all sprayed with a known amount and concentration of glucosinolate solution. Three had their test coating eluted with Milli-Q water and analysed immediately. Three were eluted after the test solution had dried (30 minutes) and the final three were analysed after 24 hours inside the bioassay test cages. An example of a chromatogram obtained for prop-*2*-enylglucosinolate is shown in Figure 24. By means of a peak height calibration graph (Fig. 25) it was possible to calculate the amount of test compound present in the eluent and therefore the amount that was present on the leaf. This was compared with the known amount originally sprayed on the leaf structure to check for any loss or degradation.

The analysis of the eluted solutions showed over 95% recovery of the original amount of the test glucosinolates applied. Allowing for some loss during the elution process this confirmed that the methanolic solution adhered to the waxy leaf surface and that there was no loss or degradation of the glucosinolates, even over a 24 hour test period.

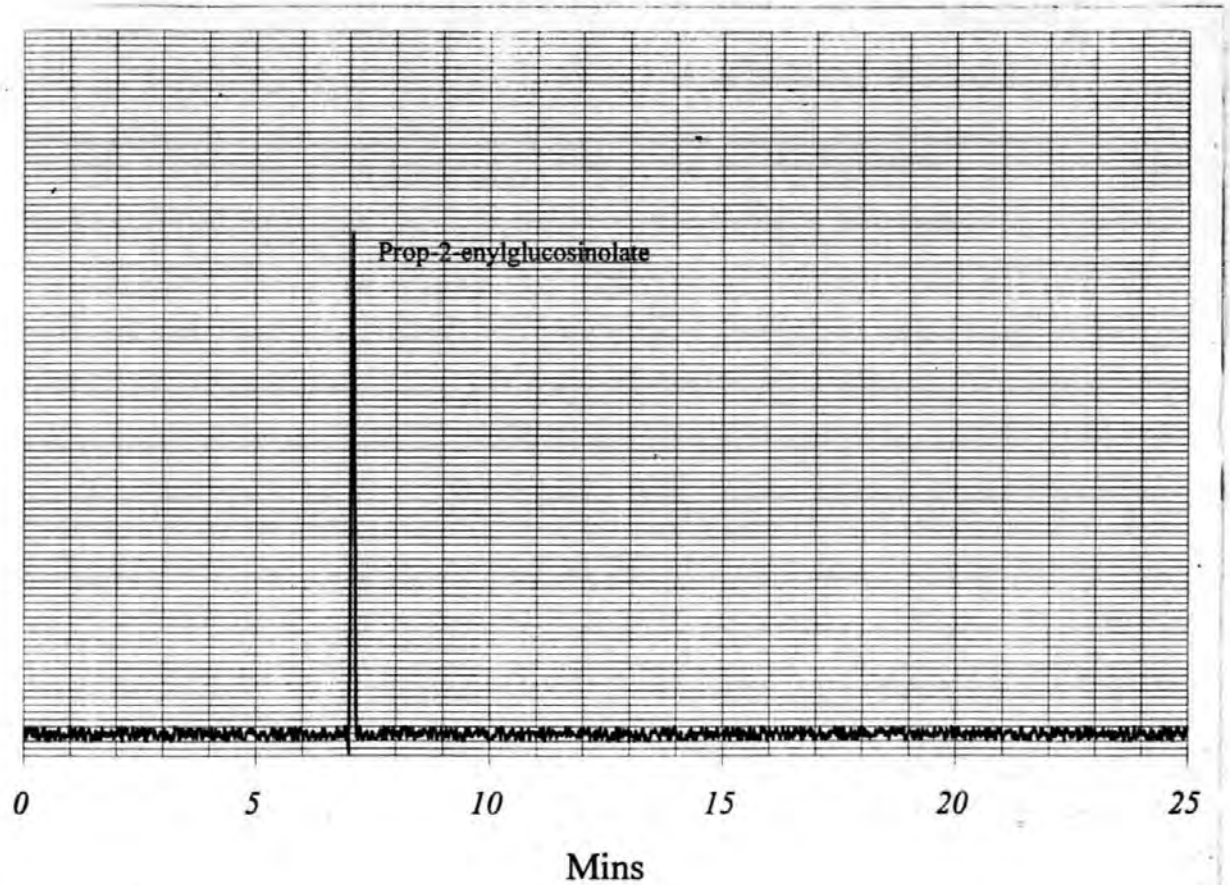


FIGURE 24 HPLC CHROMATOGRAM FOR PROP-2-ENYLGLUCOSINOLATE

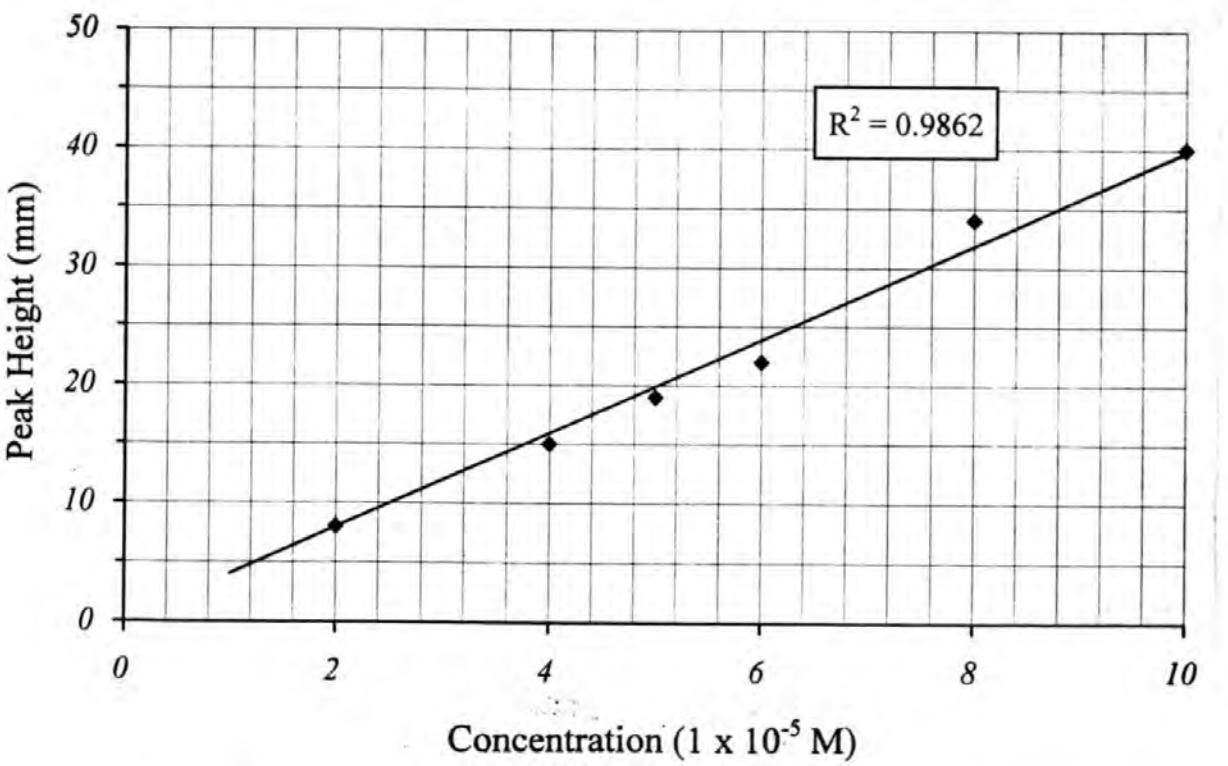


FIGURE 25 PEAK HEIGHT CALIBRATION GRAPH FOR PROP-2-ENYLGLUCOSINOLATE

In capillary electrophoresis, components of a mixture are transported through a capillary tube by a high direct current potential that is imposed over the length of the tubing. Electrophoretic separations arise from differences in mobilities of solutes from a positive electrode to a negative electrode. Electrophoretic mobility is proportional to charge on the solute and inversely proportional to the retarding forces that are determined by analyte size and shape as well as viscosity of the medium. Solvent properties such as pH, ionic strength and dielectric constant are also important because they affect the effective charge on the solvent. In 1984 Terabe and collaborators (1984) devised a capillary electrophoresis method that permitted the separation of uncharged solutes. This technique involves the introduction of a surfactant, such as sodium dodecyl sulphate at a concentration level at which micelles form. Micelles form in aqueous solutions when the concentration of an ionic species having a long-chain hydrocarbon tail is increased above a certain level called the critical micelle concentration. At this point the ions begin to aggregate and form spherical particles made up of 40 to 100 ions whose hydrocarbon tails are in the interior of the sphere and whose charged ends are exposed to the water on the outside. Micelles constitute a stable second phase that is capable of absorbing non-polar compounds into the hydrocarbon interior of the particles thus solubilising the non-polar species.

The most common surfactant used to date has been sodium dodecyl sulphate. The surface of anionic micelles of this type has a large negative charge which gives them a large electrophoretic mobility towards the positive electrode. Most buffers, however, exhibit such a large electroosmotic flow rate toward the negative electrode that anionic micelles are carried towards that electrode as well though at a much reduced rate. Thus, during an experiment, the buffer mixture consists of fast-moving aqueous phase and a slower-moving micellar phase.

When a sample is introduced to this system the components distribute themselves between the aqueous phase and the hydrocarbon phase in the interior of the micelles. The positions of the resulting equilibria depends upon the polarity of the solutes. Polar solutes favour the aqueous solution, non-polar compounds favour the hydrocarbon environment. The mechanism of separation depends upon differences in the distribution coefficients for analytes between the mobile aqueous phase and the pseudostationary phase.

The apparatus used was a Dionex capillary electrophoresis system. Micellar electrokinetic chromatography was performed in a 600 mm x 0.75 mm i.d. fused-silica capillary tube (P/N 042075). The instrument conditions included 10 second gravity injections of 100 mL of the sample solutions. Injection was at the positive end of the capillary at room temperature with an applied field strength of 10 kV, 200 μA and on-column UV detection at 229 nm. The run buffer solution comprised of Sodium Dodecyl Sulphate (0.06 M) and Borax (0.006 M) with pH adjusted to 8.92 using Boric Acid (0.006M). Capillary conditioning was obtained by flushing the capillary tube with run buffer for 2 minutes before each run.

Figure 26 is an example of a electropherogram showing the separation of prop-*2*-enylglucosinolate, but-*3*-enylglucosinolate, *2*-hydroxybut-*3*-enylglucosinolate, indol-*3*-ylmethylglucosinolate, *2*-phenylethylglucosinolate and pent-*4*-enylglucosinolate.

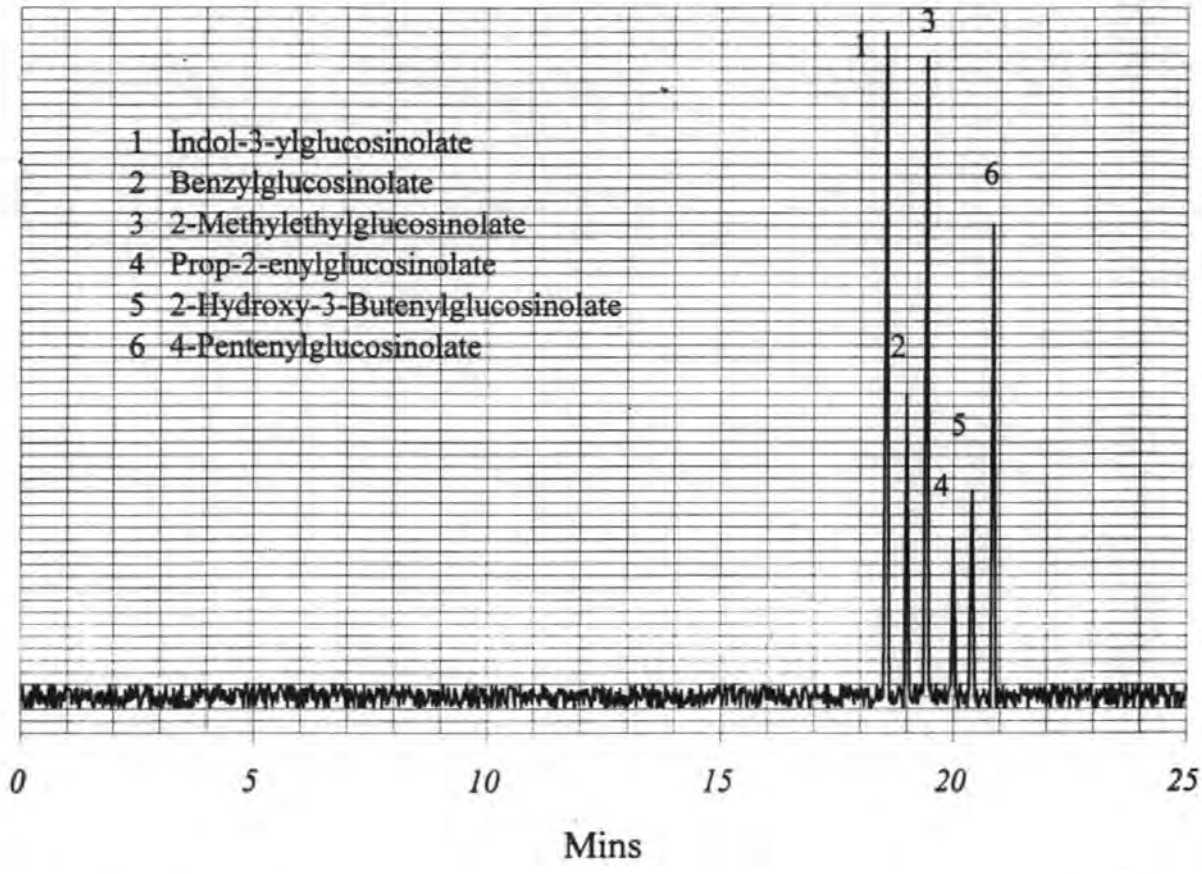


FIGURE 26 CAPILLARY ELECTROPHEROGRAM FOR A MIXTURE OF 6 GLUCOSINOLATES

2.6 DATA COLLECTION

The variation in response with the amount of test compound on the leaves was investigated over a range of concentrations. The total number of eggs laid on the standard (ΣT_1), test (ΣT_2) and blank (ΣB) sites in at each test concentration were recorded. For all concentrations there were at least two repeat bioassays.

2.7 TREATMENT OF OVIPOSITION DATA

To ensure that repeat bioassays were not statistically different, the number of eggs laid on the prop-*2*-enylglucosinolate control standards (T_2) were compared using the appropriate t-test. All repeats were found not to be statistically different at (P=5%).

A good model of the observed data of each test compound was needed to enable the prediction of the amount of each compound necessary for maximum oviposition response. Regression analysis using Microsoft Excel Version 5 produced a cubic equation relating the fly oviposition response (Y) to the $\log_{10}$ of the concentration (x) as shown below:

$$Y = ax^3 + bx^2 + cx + d$$

where:

x = $\log_{10}$ concentration (Molar)

Y = relative stimulation. $(\Sigma T_2 - \Sigma B) / (\Sigma T_1 - \Sigma B)$

with:

ΣT_1 = Total number of eggs laid on the prop-*2*-enylglucosinolate control standards

ΣT_2 = Total number of eggs laid on the surrogate leaves treated with the test compound

ΣB = Total number of eggs laid on the blanks

The optimum concentration (C) of a compound required to give maximum relative stimulation (Y_{MAX}) for each compound was then calculated by solving:

$$\frac{dY}{dx} = 0$$

i.e. $$\frac{dY}{dx} = 3ax^2 + 2bx + c = 0$$

from which x can be obtained by using the standard method for obtaining the roots of a quadratic equation:

i.e $x = \frac{-b \pm \sqrt{b^2 - 4ac}}{2a}$

The above equation would result in a positive and negative value of x although only one would be biologically possible. For those curves skewing to the left it proved to be the negative value, for those skewing to the right it was the positive value.

The resulting Y_{MAX} value ± the standard error, calculated from the repeat bioassays, was then used as a measure of biological activity. Compounds were designated as biologically active if the number of eggs laid on the treated sites was significantly higher than that on the blanks at (P=5%) using the appropriate t-test. The average number of eggs laid on the blanks was 1 though the values ranged from 0 to 3.

It had been observed that the numbers of eggs laid by the flies decreased from June through to September (Homfray, pers comm.). For this reason it was decided that a ratio between the number of eggs laid on the test sites (T_2) and the number of eggs laid on the prop-*2*-enylglucosinolate standards (T_1) would result in an accurate indication of the biological activity. The ratio would also take into account the number off eggs laid on a site by "chance" which was assumed to be equal to the number laid on the blank sites (B).

An analysis of variance test (ANOVA) was carried out to see if there was any significant difference between the number of eggs laid in each row and column of the surrogate leaves layout. For all the experiments there was no significant effect of the rows and columns in the bioassay cage at (P=5%). Examples of the all the statistical tests employed can be found in Appendix B.

3. TEST COMPOUNDS AND THEIR SYNTHESIS

3.1 GENERAL PROCEDURES

The analytical results obtained for each compound prepared can be found in Appendix C.

3.1.1 Elemental Analysis

Carbon, hydrogen, nitrogen analyses were performed by NAMAS accredited Butterworth Laboratories Ltd, Middlesex, UK, using a PE2400 instrument.

3.1.2 Fourier Transform Infra-Red Spectroscopy

Infra-red spectra were recorded using a Brüker IFS66 spectrometer. Resolution was set at 4 cm, with 32 sample scans performed between 4000 and 400 cm^{-1}. Solid samples were analysed as KBr discs, liquid samples were analysed neat using NaCl windows.

3.1.3 Gas Chromatography

Gas chromatography analyses were performed on a Carlo Erba gas chromatograph. A capillary column (length 25 m, inner diameter 0.32 mm) having a 0.25 μm DB5 coating was used. The injector temperature was maintained at 170 °C.

A sample volume of 0.5 μL of the analyte in dichloromethane (50 μgL^{-1}) was directly injected on to the column using helium as the carrier gas. The initial oven temperature was held at 40 °C for two minutes then increased to 200 °C at a rate of 7 °C min^{-1}. The purity of the samples was determined using a Shimadzu C-R3A Chromatopac integrator.

3.1.4 Gas Chromatography-Mass Spectrometry

Gas chromatography-mass spectometry was carried out using a Carlo Erba series 5160 Mega Chromatograph coupled to a Kratos MS25 double focusing magnetic sector mass spectrometer. The fused silica column (length 25 m , 0.32 mm diameter) with a 0.25 μm DB5 coating was introduced directly in to the ion source of the mass spectrometer which was maintained at 230 °C.

A sample volume of 0.5 μL of the analyte in dichloromethane (50 μgL^{-1}), was directly injected on column with helium as the carrier gas (head pressure 0.45 $Kgcm^{-2}$). The initial temperature of the oven was held at 40 °C for two minutes before being increased at an infinite rate to 100 °C. This was then followed by an increase to 300 °C at a rate of 4 °C min^{-1}.

The ionising potential was 40eV with the filament emission current 400 μA. The mass spectra were recorded in the mass range: $^{m}/_{z}$ 30 to 500, with scanning every 1.5 seconds controlled by a DS90 data system.

3.1.5 Nuclear Magnetic Resonance Spectra

The NMR spectra were recorded using a Jeol perpetual superconducting magnetic source with a current of 36 amps and a resulting magnetic field strength of 6.3 tesla. The magnet is surrounded by a liquid nitrogen bath (-169 °C) and hard vacuum.

For the proton NMR analyses a total of sixteen 4.9 microsecond pulses at a frequency of 270 MHz were sent by a Jeol JNM-EX270 FTNMR System Spectrometer with a 5 second delay between pulses to allow for thermodynamic equilibration and collection of data. Since it is a less sensitive technique, fifty pulses at a frequency of 67.8 MHz were used for the ^{13}C and DEPT NMR analyses.

All samples were dissolved in deuterated dichloromethane (approx. 10 gL^{-1}).

3.1.6 Thin Layer Chromatography

Chromatograms were produced using the ascending technique on 0.25 mm layers of either 60G silica gel or Kieselgel G.F_{254} using the following solvent systems:

A.	n-Butanol/Acetic acid/Water	60/15/25
B.	Dichloromethane/Methanol	80/20

The solutes were located using either concentrated sulphuric acid or ultra-violet absorption at 254 nm as stated.

3.2 GLASSWARE AND CHEMICALS

To avoid contamination all items were cleaned in a 10% $^{v}/_{v}$ Decon solution, washed with deionised water and dried overnight in an oven at 150 °C.

The following test compounds: thioglucose, *S*-methyl cysteine, cysteine sulphinic acid, cysteic acid, taurine, sulphamic acid, hydroxylamine-*O*-sulphonic acid, octane sulphonic acid, toluene-*p*-sulphonamide, toluene-*p*-sulphonic acid, sodium bisulphite, sodium bisulphate, mercaptopyridine-*N*-oxide, mercaptopyridine-*N*-oxide sodium salt, α–glucose-*1*-phosphate disodium salt, indole, indole acetone sodium bisulphite, indoxyl-*3*-sulphate potassium salt were purchased from Aldrich UK Ltd and used as recieved unless otherwise stated. *5*-Nitro-*2,3*-dihydro-*1,2*λ-*6*-benzoxathiole-*2,2*-dione and *1,3,2*λ-*6*-benzodioxathiole-*2,2*-dione were supplied by Bionet Ltd and Key Organics Ltd respectively. The potassium salts of three different glucosinolates were used: prop-*2*-enylglucosinolate, benzylglucosinolate and indol-*3*-ylmethylglucosinolate. Prop-*2*-enylglucosinolate was purchased from Sigma UK Ltd, the other two were provided by the Department of Biological Sciences, University of Plymouth..

3.3 PREPARATION OF MODIFIED GLUCOSINOLATES

3.3.1 Acetylation of Prop-2-enylglucosinolate

Potassium *S*-(*2,3,4,6*-tetra-*O*-acetyl-*β*-D-glucopyranosyl)-prop-*2*-enylglucosinolate was prepared accorrding to the method of Kjær and Gmelin (1956). Prop-*2*-enylglucosinolate (41.5 mg, 0.1 mmol), acetic anhydride (10.4 mL, 1 mmol) and dry pyridine (2 mL) were added to a round bottomed flask fitted with a condenser and a potassium hydroxide guard tube. The reaction mixture was stirred for 5 hours at 50 °C after which time it had turned pink in colour and a voluminous precipitate had formed. The solid was collected by filtration and dried in an evacuated dessicator over concentrated sulphuric acid to remove any residual pyridine (yield 46.4 mg, 71.2%).

The product, a white solid, chromatographed as a single spot, R_F 0.6, by thin layer chromatography using 60G silica gel and solvent system A. The solute was located by concentrated sulphuric acid. Under the same conditions prop-*2*-enylglucosinolate had a R_F of 0.27. The compound had a melting point of 194 °C (Lit. Value 194 °C; Benn and Ettlinger, 1965).

3.3.2 Desulphation of Prop-2-enylglucosinolate

S-(*β*-D-glucopyranosyl)-prop-*2*-enylthiohydroxamate was obtained by the desulphation of the glucosinolate molecule by the enzyme Aryl Sulphatase (EC 3.1.6.1) (Sigma UK Ltd). The method used was based on that of Minchinton *et al.* (1982). The enzyme (10 mg) was added to prop-*2*-enylglucosinolate (5 mg) dissolved in 50 mL of a buffer made up from acetic acid (0.2 Molar) and sodium acetate (0.2 Molar) in the ratio of 1:9 with a pH of 5.57. The solution was left overnight at 25 °C and the desulphation was considered to be complete since the sample was chromatographically homogenous

following the procedure of Spinks *et al.* (1984) with no trace of prop-*2*-enylglucosinolate present. The reaction mixture was bioassayed immediately with an appropriate blank.

3.4 SYNTHESIS OF NON-GLUCOSINOLATES

3.4.1 Acetone Sodium Bisulphite

The sodium bisulphite product of acetone was prepared according to the method of Mann and Saunders (1960). A saturated solution of sodium metabisulphite (5 mL) was shaken with the carbonyl compound (10 mL, 0.172 mol). The mixture became warm as a precipitate formed, and then, on cooling, almost solid. The product, a white solid, was collected by filtration and dried in an evacuated dessicator over phosphorus pentoxide (yield 16.4 g, 59%).

The compound had a melting point of (Lit. value °C) and was further characterised as acetone sodium bisulphite from its infra-red spectrum [*bonded* (OH) 3323 cm^{-1}, ν(C-H) *(asym)* 2962 cm^{-1}, ν(C-H) *(sym)* 2872 cm^{-1}, ρ(C-H) 1467 cm^{-1}, δ(C-H) 1375 cm^{-1}, ν(S=O) *(sym)* 1186 cm^{-1}, ν(S=O) *(asym)* 1054 cm^{-1}].

3.4.2 Cyclohexanone Oxime

Cyclohexanone oxime was prepared according to the method of Harwood and Moody (1989). Hydroxylamine hydrochloride (10 g, 0.145 mol) and sodium ethanoate (20 g, 0.148 mol) were dissolved in distilled water to which was then added the carbonyl compound (5 g, 0.05 mol). The ketone was sparingly soluble in the aqueous system so a few drops of ethanol were required to achieve the solution. The mixture was then swirled with warming for about ten minutes on a water bath and then cooled in ice. The resulting precipitate was filtered off under vacuum and recrystallised from ethanol (yield 4.86 g,

86%). The white solid was identified as the oxime from its melting point, 88 °C (Lit. value 88 °C; Mann and Saunders, 1960) and infra-red spectrum [ν(C-H) 2962 cm^{-1} and 2872 cm^{-1}, ν(C=N) 1668 cm^{-1}, ν(C=C) 1601 and 1494 cm^{-1}, ρ(C-H) 1467 cm^{-1}].

3.4.3 1,3-Diphenylacetone Oxime

The oxime was prepared as in section *3.4.2* using *1,3*-diphenylacetone (10.5 g, 0.05 mol), again a few drops of ethanol were required to dissolve the ketone fully. The *1, 3*-diphenylacetone oxime was isolated as a white crystalline solid (yield 8.31 g, 73.86%) and characterised by its melting point of 125 °C (Lit. value 125 °C; Hurd *et al.*, 1933) and infra-red spectrum [ν(C-H) 3027 cm^{-1}, ν(C=N) 1668 cm^{-1}, ν(C=C) 1602 and 1494 cm^{-1}, ρ(C-H) 1446 cm^{-1}, *monosubst* ν(C-H) 756 and 699 cm^{-1}]. δ_H ($CDCl_3$) 2.79 (2H, s) 2.96 (2H, s) 6.46 - 6.63 (10H, m, H_{Ar}).

3.4.4 1-(Toluene-4-Sulphonyl)-Indole

Indole (0.5 g, 4.3 mmol) was dissolved in a 10% $^{M}/_{V}$ sodium hydroxide solution (10 mL) to which was added a concentrated solution of p-toluene sulphonyl chloride (0.74 g, 3.9 mmol) in acetone. The mixture was shaken for 15 minutes, the resulting precipitate collected and recrystallised from methylated spirits. The compound was characterised from its melting point, 85 °C (Lit. Value 86 °C; Zheng *et al.*, 1994) and its infra-red spectrum [ν(C-H) 3031 cm^{-1}, ν(C-H) *(asym)* 2947 cm^{-1}, ν(C-H) *(sym)* 2861 cm^{-1}, ν(S=O) *(sym)* 1193 cm^{-1}, ν(S=O) *(asym)* 1052 cm^{-1}, *p-disubst* ν(C-H) 852 cm^{-1}].

3.4.5 Toluene-p-Sulphonic Acid Potassium Salt

This was prepared by titrating a solution of toluene-*p*-sulphonic acid of known concentration with an appropriate amount of potassium hydrogen carbonate solution. The pH of the solution was monitored to identify the end point.

3.4.6 S-Methyl Cysteine Sulphone

The sulphone was synthesised by the oxidation of the sulphoxide in accordance with the method of Rinderknecht *et al.*, (1958). *S*-Methyl cysteine sulphoxide (5.31 g, 35.2 mmol), ammonium molybdate (196 mg, 1 mmol) and 10.4 mL of H_2O_2 (35 % $^{v}/_{v}$) were dissolved in methanol (100 mL). The reaction mixture was stirred whilst refluxing for 4 hours. The solution was cooled, the resulting precipitate was filtered and recrystallised from ethyl ethanoate and hexane. The product, a white solid, was identified as the sulphone by its melting point , 183 °C (Lit. value 182-184 °C; Rinderknecht *et al.*, 1958) and infra-red spectrum [ν(S=O) *(sym)* 1121 cm $^{-1}$, ν(S=O) *(asym)* 1289 cm $^{-1}$].

4. GLUCOSINOLATE SYNTHESIS

4.1 BACKGROUND

There are two main synthetic routes for producing glucosinolates. One is the electrophilic approach and the other the nucleophilic. The former is the only example of the "electrophilic sugar" approach to the coupling of the aglycone and D-gluco units to appear in the literature. The method was published by Ettlinger and Lundeen (1956) after successfully elucidating the structures for prop-*2*-enylglucosinolate and *p*-hydroxybenzylglucosinolate a year earlier based on observations of the two glucosinolates' breakdown products.

The method (Fig. 27) they employed to produce the benzylglucosinolate proceeded from ethereal magnesium dithiophenylacetate which was treated with hydroxylamine hydrochloride to furnish phenylacetothiohydroxamate.

The regio- and chemoselective attack of *2,3,4,6*-tetra-*O*-acetyl-α-D-glucopyranosyl bromide on the thiohydroximate gave the *S*-(tetra-*O*-acetyl-β-D-glucopyranosyl) benzylthiohydroximate which was subsequently sulphated using pyridine-sulphur trioxide and isolated as the monohydrated potassium salt. The 'proglucosinolate' was then deprotected using methanolic ammonia.

In 1963 Benn published the "nucleophilic sugar" approach which is based on the efficient regio and stereospecific *1,3*-addition of a glycosyl mercaptan RSH on a transient nitrile oxide (Fig. 28). The unstable nitrile oxides are best prepared in *situ* by treating a hydroxamoyl chloride with base. Benn (1963; 1965) prepared the hydroxamoyl chloride by bubbling chlorine gas through a solution of the aldoxime followed by the spontaneous rearrangement of the blue chloronitroso compound formed initially.

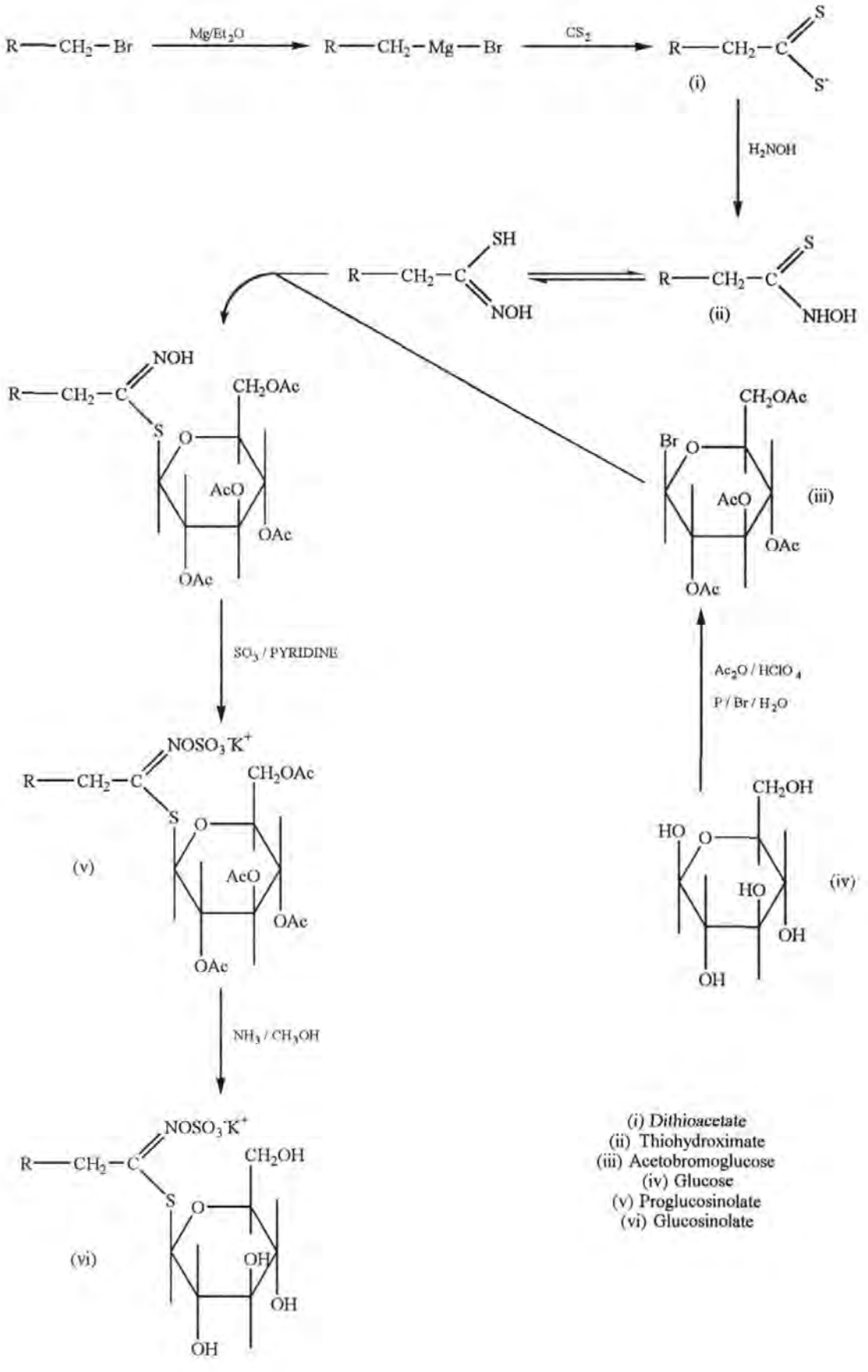


FIGURE 27 **CHEMICAL SYNTHESIS OF A GLUCOSINOLATE (ETTLINGER & LUNDEEN, 1957)**

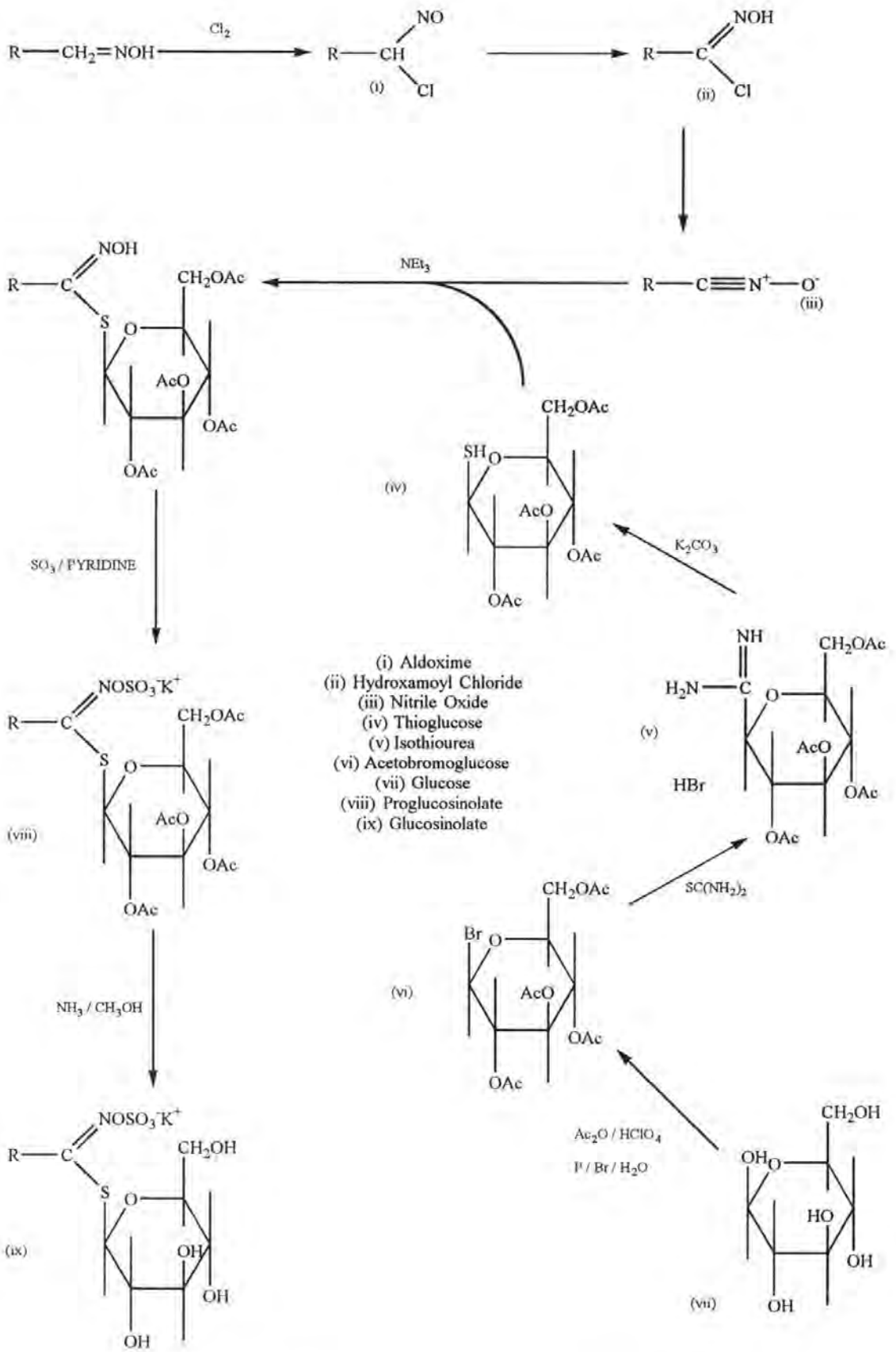


FIGURE 28 **CHEMICAL SYNTHESIS OF A GLUCOSINOLATE (BENN, 1963)**

Treatment with *1*-thio-*β*-D-glucopyranose tetra-acetate in the presence of excess triethylamine to form the glucosylthiohydroximate *via* the transient nitrile oxide. The coupling process is slightly hampered by the formation of a minor product identified as *β,β'*-(*2,3,4,6*-tetra-*O*-acetyl-D-glucopyranosyl) disulphide resulting from the oxidation of the glucosyl mercaptan.

The *O*-sulphation of the hydroximino moiety of the ester used pyridine-sulphur trioxide complex which Benn (1963) reported as an efficient reagent for this transformation.

Although other general methods are available - chloramine T oxidation of an aldoxime, hydroximoyl halide dehydrohalogenation - the preparation of the nitrile oxide by workers since Benn (Kjær and Jensen, 1968; Kjær, 1971; MacLeod and Rossiter, 1983; Viaud *et al.*, 1992; Gardrat *et al.*, 1993; Mavratzotis *et al.*, 1996) has been from a primary nitroalkyl precursor. The precursor was reacted with sodium methoxide to afford the sodium nitronate salt. Subsequent reaction at low temperatures (-30 to -40°C) with lithium chloride or thionyl chloride led to the labile hydroxamoyl chloride which was used without further purification.

Gil and Macleod (1979) modified Benn's original method and isolated the hydroxamoyl chloride by bubbling dry Cl_2 gas through a solution of the nitronate salt at low temperature (-60°C). They also commented that the commercial pyridine-sulphur trioxide complex was inadequate for producing the 'proglucosinolate' and found freshly prepared complex afforded the appropriate product in good yield and high purity.

Viaud *et al.* (1991) found that neither the bench prepared nor the commercial complex gave satisfactory results. For this reason they did not isolate freshly prepared complex but used it directly *in situ* and reported enhanced *O*-sulphating activity. The method of using pyridine and chlorosulphonic acid to produce the complex *in situ* has also

been adopted by Chevolleau *et al.* (1993) for the synthesis of radiolabelled indol-3-ylglucosinolate, by Streicher and co-workers (1995) for their syntheses of deoxy derivatives of glucosinolates and by Mavaratzotis *et al.* (1996) for the synthesis of glucosinolates with an external thio-function.

4.2 SYNTHETIC PATHWAY 1

4.2.1 Overview

Despite the instability of most thiohydroximates (Walter and Schaumann, 1971) it was decided to investigate this pathway first as the thiohydroximates are also intermediates in the biosynthesis of glucosinolates and therefore it would be sensible to attempt to synthesise and bioassay them in their own right.

The carboxymethyl dithioacetate was prepared according to the method of Kjær (1952) by treating benzylmagnesium chloride with carbon disulphide in ether. The conversion to the thiohydroximate followed the method published by Walter and Schaumann (1971) with the dithioacetate added to a solution of hydroxylamine hydrochloride and sodium carbonate. Sulphur which formed was removed by filtration and the crude product separated out as an oil on acidification of the reaction solution. Recrystallisation of the isolated crude product afforded the thiohydroximate which decomposed overnight to an amorphous yellow solid. An infra-red spectrum showed peaks between 2130 and 2170 cm^{-1} which are caused by the asymmetrical stretching of the N=C=S system and are characteristic of an isothiocyanate.

A "one-pot" synthesis of phenylacetothiohydroximate using the method of Davies *et al.* (1968) afforded the thiohydroximate which was, again, observed to decompose over the space of one hour to an amorphous yellow solid.

Infra-red monitoring showed that the compound was undergoing rearrangement to an isothiocyanate as indicated by an increase over time in the intensity of the characteristic peaks between 2130 and 2170 cm^{-1}. It is thought that the rearrangement was similar to the Lossen rearrangement that occurs during the breakdown of glucosinolates. A reaction mechanism is shown in Fig. 29.

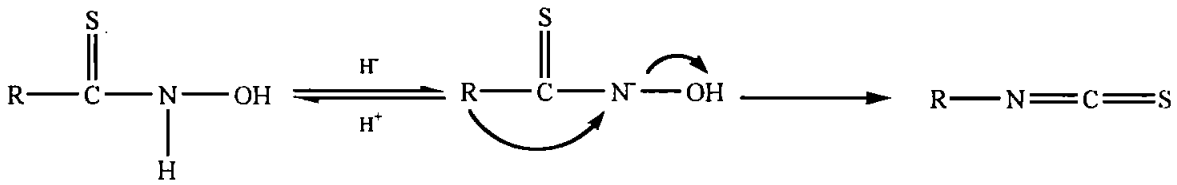


FIGURE 29 LOSSEN REARRANGEMENT

4.2.2 Synthesis of Phenylacetothiohydroximate

4.2.2.1 Preparation of Carboxymethyl Dithiophenylacetate

The Grignard solution was prepared under a nitrogen atmosphere by adding benzyl chloride (37.5 mL, 0.325 mol) to dried magnesium turnings (8.25 g, 0.34 mol) in sodium dried diethyl ether (150 mL). When the reaction subsided the mixture was gently heated to ensure completion. The ether solution was then decanted off and added over the space of an hour to a stirred, ice-cooled solution of dry carbon disulphide (30 mL, 0.5 mol) in dry diethyl ether (75 mL). The mixture was kept overnight after which time it was poured onto 200 g of crushed ice and the aqueous layer separated off from the ethereal layer which contained an amorphous precipitate. The addition to the aqueous portion of a solution containing chloroacetic acid (32 mL, 0.34 mol) and sodium carbonate (19 g, 0.18 mol) in 175 mL of water resulted in the slow formation of fine yellow needles, the sodium salt of carboxymethyl dithiophenylacetate. After storing for 48 hours at 0 °C the free dithioacid

was liberated as a brown oil by the dropwiseaddition of 22 mL of concentrated sulphuric acid (0.415 mol) and 25 mL of water. The oil was extracted into diethyl ether which was then dried over sodium sulphate and the ether evaporated under reduced pressure leaving a red oil. Recrystallisation from petroleum ether (40-60 °C) gave rise to yellow crystals and an oil. The crystalline solid was recovered by filtration, washed with cold petroleum ether and identified as carboxymethyl dithiophenylacetate from its melting point, 79 °C (Lit. value 79 °C; Kjær, 1952) and infra-red spectrum. The remaining oil was washed with portions of sodium hydrogen carbonate until the release of carbon dioxide ceased. The solution was then acidified with hydrochloric acid to congo red with the oil released extracted into diethyl ether, dried over sodium sulphate and evaporated under reduced pressure. The resulting yellow crystalline solid was recrystallised from petroleum ether and identified as the dithioacetate as before. Total yield 44.3 g (65%)

4.2.2.2 Preparation of Phenylacetothiohydroximate

A solution of carboxymethyl dithiophenylacetate (10 mmol) in 1 Molar sodium hydroxide (10 mL) was added, with cooling, to a solution of hydroxylamine hydrochloride (0.76 g; 11 mmol) in 1 Molar sodium hydroxide (11 mL) keeping the pH at pH 7. The reaction mixture was stirred for 24 hours before being acidified with acetic acid. The crude product was extracted with diethyl ether. The combined extracts were dried with magnesium sulphate, filtered and evaporated under vacuum. The residue was recrystallised using petroleum ether (40-60 °C) to give long thin white crystals of phenylacetothiohydroximate (yield 1.276 g; 76%) identified by its melting point 74 °C (Lit. value 73-74 °C; Walter and Schaumann, 1971). The product was stored overnight at 0 °C under vacuum during which time degradation to a yellow amorphous solid had taken place.

4.2.3 "One-pot" Synthesis of Phenylacetothiohydroximate

Magnesium dithiophenylacetate chloride was synthesised as described in section *4.1.2* except the preparation was stopped after the addition of carbon disulphide to the benzyl magnesium chloride. The ether solution was chilled and was immediately added dropwise to an ice-cooled solution of hydroxylamine hydrochloride (22 g, 0.33 mol) and sodium carbonate (17 g, 0.16 mol) in 250 mL of water. During this addition the magnesium salt went into solution, the ether layer became brown and hydrogen sulphide was evolved. A yellow precipitate formed in the aqueous layer which was collected by filtration and identified as sulphur by its melting point, 111 °C (Lit. value 112 °C). The product was separated from the aqueous phase by extraction with diethyl ether which was then dried over calcium chloride and evaporated under reduced pressure to give an oil residue. The oil was washed with petroleum ether (40-60 °C) to give long thin white crystals of phenylacetothiohydroximate identified by its melting point as before.

The crystals were observed to degrade over the space of one hour into a yellow amorphous solid with a distinct aroma.

4.3 SYNTHETIC PATHWAY 2

4.3.1 Overview

The bromoalkyl precursor *2*-bromoethylbenzene was nitrated to form the corresponding primary nitro compound which, when reacted with sodium methoxide following the literature method of Viaud and co-workers (1991) as shown in Figure 30, produced the sodium nitronate salt.

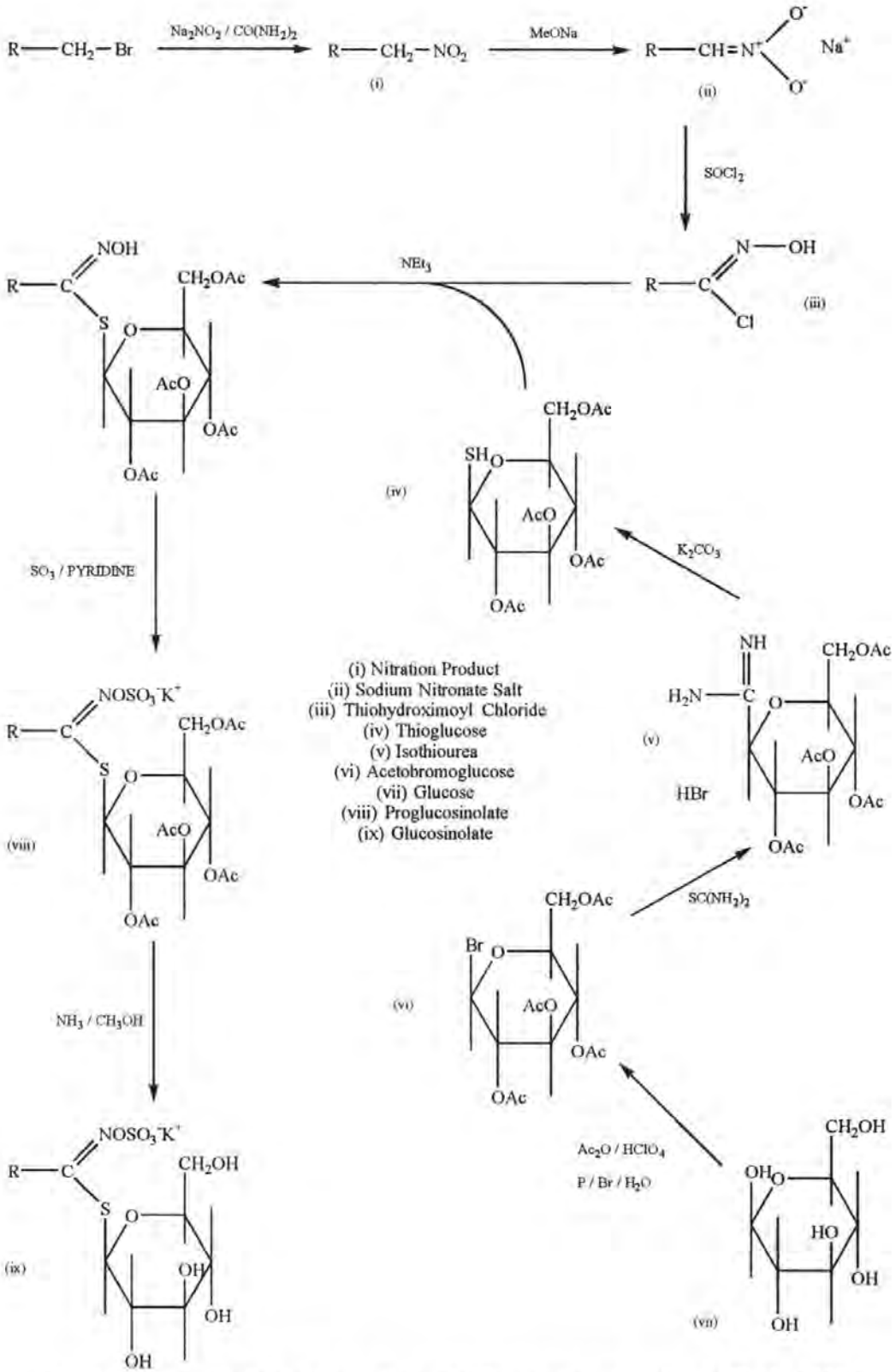


FIGURE 30 **CHEMICAL SYNTHESIS OF A GLUCOSINOLATE (VIAUD *ET AL.*, 1991)**

Attempts to prepare the thiohydroximate according to the method of Viaud *et al.* (1991) were unsuccessful with only nitronate salt recovered. *3,5*-Dimethylthiophenol was

employed as a convenient substitute for the *2,3,4,6*-tetra-*O*-acetyl-*1*-thio-*β*-D-glucopyranose to investigate the procedure. The resulting modifications to the literature method included the use of a greater volume of *1,2*-dimethoxymethane solvent to produce a solution of the nitronate salt instead of using a suspension. To reduce the risk of the labile thiohydroximoyl chloride degrading before the addition of the thiophenol the dichloromethane extract was only dried over magnesium sulphate and used immediately, it was not evaporated under reduced pressure and redissolved in anhydrous diethyl ether as stated in the literature. Treatment with *3,5*-dimethylthiophenol in the presence of triethylamine at -25 °C, not room temperature, afforded the crude *S*-(*3,5*-dimethylphenyl)benzylthiohydroximate in dichloromethane. Evaporation under reduced pressure and the use of hexane to wash the crude residue and remove any impurities led to the isolation of the desired *S*-(*3,5*-dimethylphenyl)benzylthiohydroximate.

The attempted sulphation of the *S*-(*3,5*-dimethylphenyl)benzylthiohydroximate according to the method of Viaud *et al.* (1991) was unsatisfactory. NMR analysis of the resulting crude residue showed the presence of peaks corresponding with the desired product. However what small amount of product there might have been quickly degraded during purification by column chromatography.

The synthesis of *S*-(tetra-*O*-acetyl-*β*-D-glucopyranosyl) benzylthiohydroximate following the method of Viaud *et al.* (1991) and incorporating the modifications as described above was successful, although attempts to then sulphate the thiohydroximate failed. Unreacted starting material was recovered from the dichloromethane fraction and analysis of the aqueous fraction also showed no sign of any expected product.

Further attempts were made to sulphate the *S*-(tetra-*O*-acetyl-*β*-D-glucopyranosyl) benzylthiohydroximate using both commercially available and freshly prepared pyridine-

sulphur trioxide complex. Reactions with both of these failed to produce the desired product and again only unreacted starting materials could be isolated.

Sulphation of the oxime group of *1,3*-diphenylacetone oxime to investigate the procedure and produce a compound more strictly analogous to a glucosinolate was unsuccessful, the reaction producing an amide by a spontaneous Beckmann rearrangement of the desired product (Fig. 31). This result was not unexpected.

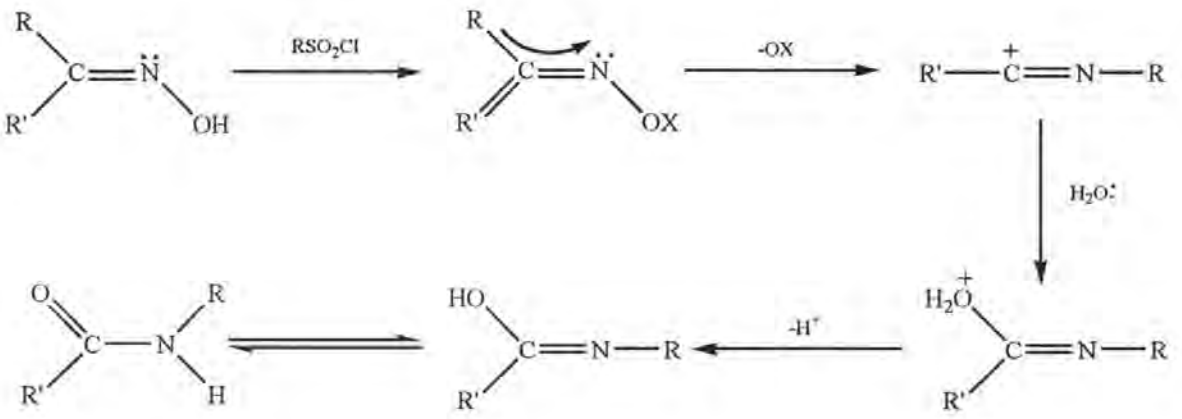


FIGURE 31 SULPHATION OF AN OXIME AND SUBSEQUENT BECKMANN REARRANGEMENT TO AN AMIDE

4.3.2 Synthesis of S-(3,5-Dimethylphenyl) Benzylthiohydroximate

4.3.2.1 Preparation of 2-Nitroethylbenzene

The nitro compound was prepared according to the method of Kjær and Jensen (1968). To a solution of sodium nitrite (34.9 g, 0.5 mol) and urea (34.5 g, 0.57 mol) in dry dimethyl sulphoxide (313 mL) was added *2*-bromoethylbenzene (38 mL, 0.27 mol). Care was taken to exclude water from the apparatus and therefore minimise the formation of any alcohol produced by the hydrolysis of the nitrite ester. The exothermic reaction was

controlled with slight cooling and the solution stirred at room temperature for 6 hours. The resulting brown solution was then poured into water (750 mL). The crude product was extracted into petroleum ether (40-60 °C), dried over sodium sulphate and the solvent removed by evaporation under reduced pressure. Any alcohol that had formed was then removed by shaking the residual oil with water and drying over sodium sulphate. An infra-red spectrum of the crude product showed peaks for both the RNO_2 [ν(N=O) 1554 and 1382 cm^{-1}] and RON=O [*cis* ν(N=O) 1620 cm^{-1} and *trans* ν(N=O) 1680 cm^{-1}]. No peaks corresponding to the alcohol were present. Analysis by gas chromatography showed two peaks (retention times 16.7 mins and 21.3 mins) although neither corresponded to the bromo starting material.

Fractional distillation of the crude product afforded the colourless 2-nitroethylbenzene (yield 12 mL, 33%) with boiling point 74 °C/0.3 torr. The compound was pure by gas chromatography (retention time 21.3 mins) and an infra-red spectra showed only peaks corresponding to the monosubstituted aromatic -NO_2 compound [ν(C-H) 3040 cm^{-1}, [ν(N=O) 1554 and 1382 cm^{-1}, ν(C=C) 1500 cm^{-1}, ρ(C-H) 1450 cm^{-1}, *monosubst* ν(C-H) 750 and 705 cm^{-1}]. Analysis by NMR found clearly assignable peaks. δ_H ($CDCl_3$) 3.26 (2H, t) 4.55 (2H, t) 7.16 - 7.33 (5H, m, H_{Ar}). (Found: C 63.37%; H 6.08%; N 9.27%; O 23.31%. Expected for $C_8H_9NO_2$: C 63.57%; H 5.96%; N 9.27%; O 21.2%).

4.3.2.2 Preparation of Sodium Nitronate Salt

To a stirred solution of sodium methoxide (2.7 g, 50 mmol) in methanol was added *2*-nitroethylbenzene (7.55 g, 50 mmol). The mixture was stirred for 10 minutes before dry diethyl ether (400 mL) was added resulting in the precipitation of the nitronate salt (yield

7.95g, 92.5%). The crude salt was collected by filtration and dried in a dessicator at 0 °C over phosphorus pentoxide under vacuum and used without further purification.

4.3.2.3 Preparation of the S-(3,5-Dimethylphenyl) Benzylthiohydroximate

The dried and finely ground nitronate salt (1.9 g, 0.011 mol) was added rapidly under nitrogen to cooled (-40 °C) *1,2*-dimethoxymethane (60 mL). To the resulting suspension was added a solution of thionyl chloride (3 mL, 0.042 mol) in dimethoxymethane (20 mL) via a syringe with stirring. After stirring for 30 minutes at -40 °C the mixture was treated with water (100 mL) and the excess *1,2*-dimethoxymethane evaporated under reduced pressure at room temperature. The resulting mixture was then extracted with dichloromethane, dried over magnesium sulphate and used in the next step without purification.

To a stirred solution of the crude hydroximoyl chloride under nitrogen at -25 °C were successively added *3,5*-dimethylthiophenol (18 mL, 0.013 mol) in dry dichloromethane (20 mL) and fresh triethylamine (5 mL, 0.037 mol). After stirring for 1 hour at -25 °C the mixture was washed twice with sulphuric acid (1 Molar) and twice with water. The dichloromethane layer was then dried over magnesium sulphate and evaporated to dryness. The remaining residue was washed with hexane to remove impurities leaving a crystalline solid (yield 1.39 g, 54%) with melting point 110 °C. The NMR and elemental analyses yielded the following results. δ_H ($CDCl_3$) 2.19 (6H, s) 3.51 (2H, s) 6.81 - 7.25 (8H, m, H_{Ar}). (Found: C 70.28%; H 6.18%; N 5.26%. Expected for $C_{16}H_{17}NOS$: C 70.85%; H 6.27%; N 5.17%).

4.3.2.4 Attempted Sulphation of S-(3,5-Dimethylphenyl) Benzylthiohydroximate

To a cooled (0 °C) solution of pyridine (2 mL) in dry dichloromethane (5 mL) under nitrogen, a solution of chlorosulphonic acid (0.6 mL, 9.4 mmol) in dry dichloromethane (5 mL) was added via a syringe over a period of 10 minutes followed by the introduction of a solution of *S*-(*3,5*-dimethylphenyl) benzylthiohydroximate (254 mg, 0.94 mmol) in dry dichloromethane (4 mL) also via a syringe. After 24 hours at room temperature the medium was hydrolysed with a solution of potassium hydrogen carbonate (470 mg, 4.3 mmol) in water (10 mL), stirred for another 30 minutes and then extracted with dichloromethane (3 x 10 mL). The extracts were combined and dried over magnesium sulphate and evaporated to dryness. NMR analysis of the crude residue showed peaks corresponding to those expected for the desired product. δ_H ($CDCl_3$) 2.00 (6H, s) 3.5 (2H, s) 5.28 (1H, s, H_{Ar}) 6.53 - 6.93 (7H, m, H_{Ar}) 7.29 - 8.72 (m, $H_{Pyridine}$). The small amount of product that was present was lost during purification by column chromatography using 30% methanol : 70% dichloromethane solvent system.

4.3.3 Synthesis of S-(Tetra-O-Acetyl-ß-D-Glucopyranosyl) Benzylthiohydroximate

4.3.3.1 Preparation of 2,3,4,6-Tetra-O-Acetyl-α-D Glucopyranosyl Bromide

The *2,3,4,6*-tetra-*O*-acetyl-α-D glucopyranosyl bromide was prepared according to the method of Vogel (1971). Acetic anhydride (100 mL, 1.06 mol) was cooled to 0 °C in a three necked flask fitted with a mechanical stirrer to which was then added, dropwise, 60% perchloric acid (0.6 mL). The mixture was then allowed to warm to room temperature and dry powdered α-D-glucose (25 g, 0.14 mol) was added in portions with stirring so that the temperature remained between 30 and 40 °C. The solution was cooled to 15 °C and, with stirring, red phosphorus (8 g, 0.25 mol) added followed by bromine (14.5 mL, 0.59 mol)

dropwise at a rate which kept the temperature below 20 °C. Then water (9 mL) was added with stirring over the period of 30 minutes whilst maintaining the below 20 °C. The reaction mixture was then left at room temperature for 2 hours before being diluted with dichloromethane (75 mL) and filtered through a funnel with a glass wool plug inserted in the outlet. The filtrate was washed with two portions of iced water (2 x 100 mL). The organic layer was then run into a saturated solution of aqueous sodium hydrogen carbonate. After the evolution of carbon dioxide had ceased the dichloromethane layer was separated off, dried with magnesium sulphate and the solvent then evaporated under vacuum. Portions of the remaining crystalline residue were then ground up with petroleum ether (40-60 °C) with the resulting slurry filtered off, washed with dry, ice cold diethyl ether. The product was then recrystallised from petroleum ether (40-60 °C) to give a crystalline solid (yield 47 g, 82%) with a melting point of 88 °C (Lit. value 88-89 °C; Vogel, 1971).

4.3.3.2 Preparation of 2-(2,3,4,6-Tetra-O-Acetyl-ß-D-Glucopyranosyl)-2-Thiopseudourea Hydrobromide

The thiopseudourea hydrobromide was prepared according to the method of Horton (1976). Thiourea (6 g, 0.078 mol) and *2,3,4,6*-tetra-*O*-acetyl-α-D glucopyranosyl bromide (30 g, 0.079 mol) were dissolved in acetone (30 mL) and refluxed for 15 minutes. Crystallisation of the product occurred during the last 5 minutes of the refluxing and was completed by cooling the solution in ice. The crystals were filtered and washed with ice cold acetone. The crude product (yield 31 g, 83 %) was used without further recrystallisation. The melting point of the crude material was found to be 179 °C (Lit. value 178 °C; Horton, 1976)

4.3.3.3 Preparation of 2,3,4,6-Tetra-O-Acetyl-1-Thio-β-D Glucopyranose

2,3,4,6-tetra-*O*-acetyl-*1*-thio-*β*-D glucopyranose was prepared according to the method of Horton (1976). A solution of *2*-(*2,3,4,6*-tetra-*O*-acetyl-*β*-D-glucopyranosyl)-*2*-thiopseudourea hydrobromide (29 g, 0.06 mol) in water (120 mL) was treated with a saturated aqueous solution of 12 g of potassium carbonate. After stirring for 30 minutes at room temperature the product had precipitated out and was collected by filtration and recrystallised. The product (yield 22 g; 77 %) was identified by its melting point of 75 °C (Lit. value 75 °C; Horton, 1976)

4.3.3.4 Preparation of S-(Tetra-O-acetyl-β-D-Glucopyranosyl) Benzylthiohydroximate

The dried and finely ground sodium nitronate salt of *2*-nitroethylbenzene was added rapidly (1.9 g, 0.011 mol) under nitrogen to cooled (-40 °C) dimethoxymethane (60 mL). To the resulting suspension was added a solution of thionyl chloride (3 mL, 0.042 mol) in dimethoxymethane (20 mL) via a syringe with stirring. After stirring for 30 minutes at -40 °C the mixture was treated with water (100 mL) and the excess dimethoxymethane evaporated under reduced pressure at room temperature. The resulting mixture was then extracted with dichloromethane, dried over magnesium sulphate and used in the next step without purification.

To a stirred solution of the crude hydroximoyl chloride under nitrogen at -25 °C were successively added the *2,3,4,6*-tetra-*O*-acetyl-*1*-thio-*β*-D-glucopyranose (5 g, 0.013 mol) in dry dichloromethane (20 mL) and fresh triethylamine (5 mL, 0.037 mol). After stirring for 1 hour at -25 °C the mixture was washed twice with sulphuric acid (1 Molar) and twice with water. The dichloromethane layer was then dried over magnesium sulphate and evaporated to dryness. The remaining residue was washed with hexane to remove impurities leaving a crystalline solid (yield 3.4 g, 46%). The white solid was characterised

by its melting point of 165 °C (Lit. value 164.1 °C; Ettlinger and Lundeen, 1957). The structure was confirmed by elemental analysis (Found: C 53.08; H 5:51; N 2.78. Expected for $C_{22}H_{27}NO_{10}S$: C 53.12%; H 5.43%; N 2.82%).

4.3.3.5 Attempted Sulphation of S-(Tetra-O-Acetyl-β-D-Glucopyranosyl) Benzylthiohydroximate

To a cooled (0 °C) solution of pyridine (4.2 mL) in dry dichloromethane (10 mL) under nitrogen, a solution of chlorosulphonic acid (1.26 mL, 19.7 mmol) in dry dichloromethane (10 mL) was added via syringe over a period of 10 minutes. This was followed by the addition of a solution of *S*-(tetra-*O*-acetyl-*β*-D-glucopyranosyl) benzylthiohydroximate (1 g, 19.7 mmol) in dry dichloromethane (8 mL) also via syringe. After 24 hours at room temperature the medium was hydrolysed with a solution of potassium hydrogen carbonate (987 mg, 9.66 mmol) in water (20 mL), stirred for another 30 minutes and then extracted with dichloromethane (3 x 20 mL). The extracts were combined and dried over magnesium sulphate.

Analysis of the extracts by thin layer chromatography using 60G silica gel and solvent system A identified the presence of unreacted starting material as a single spot R_F 0.74. The plate was visualised using concentrated sulphuric acid The dried dichloromethane extracts were evaporated to dryness and the resulting residue purified by column chromatography using the 30% methanol : 70% dichloromethane solvent system. The resulting white solid was characterised as unreacted starting material by its melting point of 165 °C (Lit. value 164.1 °C; Ettlinger and Lundeen, 1957).

Analysis by thin layer chromatography of the aqueous reaction mixture showed no signs of expected product.

Sulphation attempts using both commercial and freshly prepared pyridine-sulphur trioxide complex (Fieser and Fieser, 1967) instead of chlorosulphonic acid afforded similar results with the recovery of unreacted starting material. Analysis of the aqueous reaction mixtures and the dichloromethane extracts by thin layer chromatography showed no sign of expected product in each case.

4.3.3.6 Preparation of S-(β-D-Glucopyranosyl) Benzylthiohydroximate

S-(*2,3,4,6*-tetra-*O*-acetyl-*β*-D-glucopyranosyl) benzylthiohydroximate (500 mg, 1 mmol) was dissolved in anhydrous methanol, saturated with ammonia (15 mL) and kept at 0 °C overnight. The reaction mixture was evaporated to dryness under reduced pressure and the residue extracted with diethyl ether, taken up into water and freeze dried to yield *S*-(*β*-D-glucopyranosyl) benzylthiohydroximate as a white amorphous powder (yield 352 mg, 70.4 %) which was chromatographically homogeneous when examined on 60G gel with solvent system A. The solute was located by concentrated sulphuric acid as a single spot R_F 0.56. Under the same conditions the starting material had a R_F of 0.74. The compound was characterised by its melting point 184°C (Lit. value 183°C; Benn, 1963) and chromatographically using the method of Spinks *et al.*, 1984)

4.3.4 Sulphation of an Oxime

4.3.4.1 Attempted Sulphation of 1,3-Diphenylacetone Oxime

A solution of chlorosulphonic acid (0.6 mL, 0.01 mol) in dry dichloromethane (5 mL) was added to a cooled (0 °C) and stirred solution of pyridine (2 mL, 0.025 mol) in dry dichloromethane (5 mL) over a period of 5 minutes followed by the addition of a solution of the oxime (225 mg, 0.001 mol) in dry dichloromethane (4 mL). The reaction mixture was then stirred at room temperature for 24 hours after which time the medium was hydrolysed

with a solution of potassium hydrogen carbonate (470 mg, 0.005 mol) in water (10 mL). After a further 30 minutes stirring, the product was extracted with dichloromethane, the extracts dried and evaporated under reduced pressure to leave a crystalline solid. NMR analysis showed the presence of peaks corresponding to an amide, the result of Beckmann rearrangement. δ_H ($CDCl_3$) 3.44 (2H, s) 3.65 (2H, s) 7.15 - 7.32 (10H, m, H_{ar}) 8.98 (1H, s, H_{OH}).

4.4 SUMMARY

The attempts to synthesise phenylacetothiohydroximate were successful and fine crystals of the desired product were isolated. It would have been interesting to bioassay the compounds as thiohydroximates which are also intermediates in the biosynthesis of glucosinolates, however as with most thiohydroximates (Walter and Schaumann, 1971) the compound proved to be unstable undergoing a Lossen type rearrangement to an isothiocyanate.

Modifications to the method of Viaud *et al.* (1992) were required to prevent the decomposition of intermediate compounds. These involved working at lower temperatures and the removal of unnecessary extraction and isolation procedures.

Attempts to synthesise potassium *2,3,4,6*-tetra-*O*-acetyl-*3*-benzylglucosinolate following the method of Viaud *et al.* (1991) were unsuccessful and only unreacted thiohydroximate was recovered. Care was taken to exclude all moisture from the apparatus and reagents in addition to careful control of the reaction temperature.

Further attempts to sulphate the thiohydroximate using both commercially available and freshly prepared pyridine-sulphur trioxide complexes also failed to produce the desired product with only unreacted starting materials being isolated.

Pyridine-sulphur trioxide complex (either pre-prepared or produced *in situ*) is commonly used for the successful and facile sulphation of carbohydrates (Kennedy, 1988). There are no known reasons why this should not also have been the case for the *S*-(tetra-*O*-acetyl-β-D-glucopyranosyl) benzylthiohydroximate.

As expected sulphation of the oxime group of cyclohexanone and *1,3*-diphenylacetone oximes to produce compounds more strictly analogous to glucosinolates were unsuccessful. The oximes OH's were converted into a good leaving groups and the reactions produced amides by a spontaneous Beckmann rearrangement of the desired products.

5. BIOASSAY RESULTS AND DISCUSSION

Oviposition data for all the bioassays can be found in Appendix A. Altogether 381 bioassays involving 15, 852 eggs were carried out.

Table 6 shows the structures, optimum concentrations (C) and maximum stimulation relative to the 2.5 x 10^{-5} M prop-*2*-enylglucosinolate (Y_{MAX}) for all the compounds bioassayed presented in the approximate order of the study as it developed.

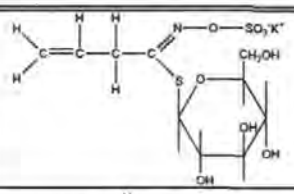
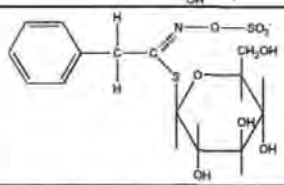
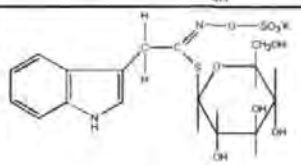
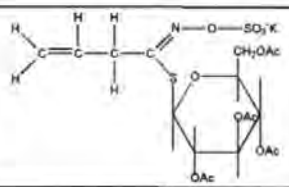
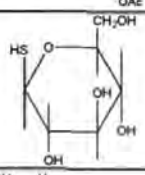
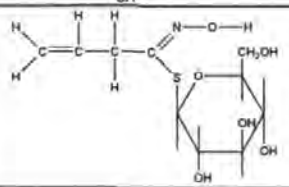
No.	Structure	Compound Name	C (Molar)	Y_{MAX} ± Std Error
1		Prop-*2*-enylglucosinolate	3.8 x 10^{-4}	2.82 ± 0.16
2		Benzylglucosinolate	2.2 x 10^{-6}	1.83 ± 0.13
3		Indol-*3*-yl methyl glucosinolate	1.4 x 10^{-5}	5.30 ± 0.29
4		Potassium *2,3,4,6*-tetra-*O*-acetyl prop-*2*-enylglucosinolate	5.5 x 10^{-5}	1.17 ± 0.06
5		Thioglucose	Not Active	Not Active
6		*S*-(β-D-Glucopyranosyl)-prop-*2*-enylthiohydroximate	Not Active	Not Active

TABLE 6 NAMES, STRUCTURES AND DETAILS OF ACTIVITY FOR ALL COMPOUNDS BIOASSAYED

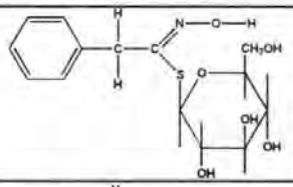
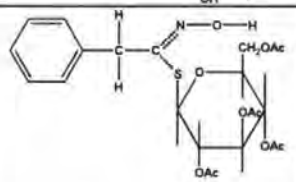
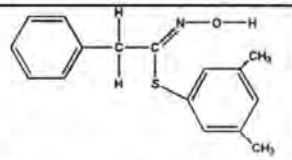
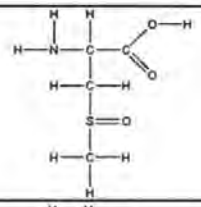
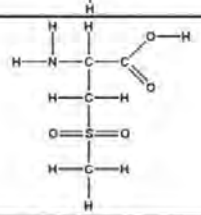
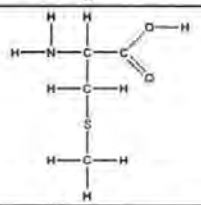
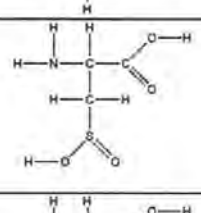
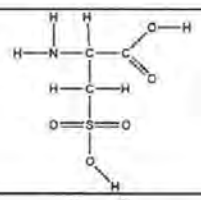
7		*S*-(*β*-D-Glucopyranosyl)-benzylthiohydroximate	Not Active	Not Active
8		*S*-(*2,3,4,6*-Tetra-*O*-acetyl-*β*-D-glucopyranosyl)-benzylthiohydroximate	Not Active	Not Active
9		*S*-(*3,5*-Dimethylphenyl)-benzylthiohydroximate	Not Active	Not Active
10		*S*-Methyl cysteine sulphoxide	1.44×10^{-6}	1.16 ± 0.06
11		*S*-Methyl cysteine sulphone	4.11×10^{-6}	1.37 ± 0.06
12		*S*-Methyl cysteine	Not Active	Not Active
13		Cysteine sulphinic acid	1.59×10^{-6}	1.28 ± 0.05
14		Cysteic acid	1.97×10^{-6}	1.57 ± 0.07
15		Taurine	1.16×10^{-6}	1.63 ± 0.07

TABLE 6 CONTINUED

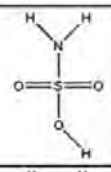
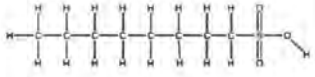
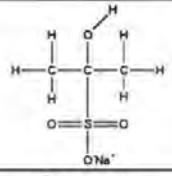
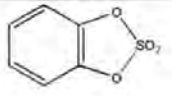
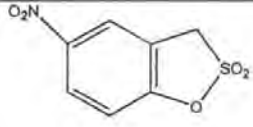
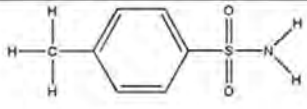
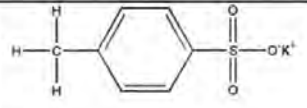
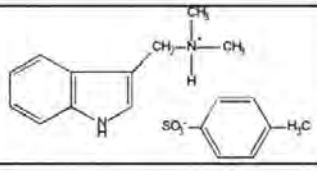
16		Sulphamic acid	4.99×10^{-5}	2.01 ± 0.15
17		Hydroxylamine-*O*-sulphonic acid	4.39×10^{-5}	1.67 ± 0.06
18		Octane sulphonic acid	3.19×10^{-6}	1.29 ± 0.08
19		Acetone sodium bisulphite	4.29×10^{-4}	0.70 ± 0.06
20		*1,3,2λ-6*-Benzodioxathiole-*2,2*-dione	1.01×10^{-4}	1.42 ± 0.05
21		*5*-Nitro-*2,3*-dihydro-*1,2λ-6*-benzoxathiole-*2,2*-dione	1.10×10^{-6}	0.98 ± 0.07
22		Toluene-*p*-sulphonamide	1.37×10^{-6}	1.12 ± 0.06
23		Toluene -*p*-sulphonic acid	9.25×10^{-5}	1.10 ± 0.1
24		Potassium toluene-*p*-sulphonate	1.70×10^{-5}	0.97 ± 0.05
25		Gramine toluene-*p*-sulphonate	7.94×10^{-4}	0.63 ± 0.05
26	$NaHSO_3$	Sodium bisulphite	Not Active	Not Active
27	$NaHSO_4$	Sodium bisulphate	Not Active	Not Active

TABLE 6 CONTINUED

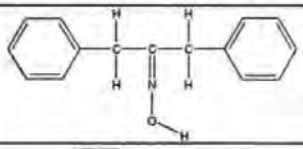
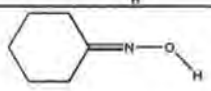
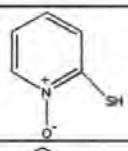
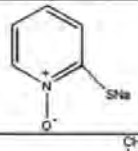
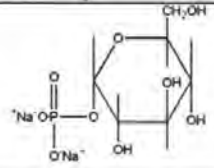
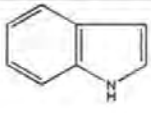
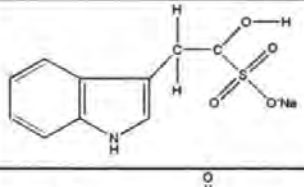
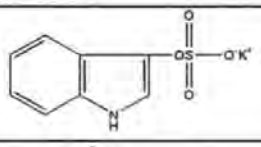
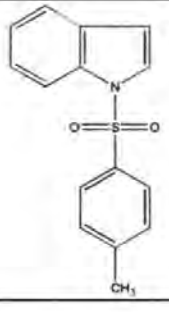
28		*1,3*-Diphenylacetone oxime	Not Active	Not Active
29		Cyclohexanone oxime	Not Active	Not Active
30		*2*-Mercaptopyridine-*N*-oxide	Not Active	Not Active
31		*2*-Mercaptopyrine-*N*-oxide sodium salt	Not Active	Not Active
32		α-D-Glucose-*1*-phosphate, disodium salt	Not Active	Not Active
33		Indole	Not Active	Not Active
34		Indole-*3*-acetaldehyde sodium bisulphite	4.63×10^{-6}	1.07 ± 0.07
35		Indoxyl-*3*-sulphate, potassium salt	2.25×10^{-4}	3.43 ± 0.2
36		*1*-(Toluene-*4*-sulphonyl)-indole	1.52×10^{-6}	0.66 ± 0.05

TABLE 6 CONTINUED

Figures 32 to 53 each show a plot of relative stimulation (Y) versus $\log_{10}$ concentration (x) for the active compounds tested. Each plot shows the data points with error bars of ± the standard error along with the best fit curves, their R-squared values and the equations of the line from which C and Y_{MAX} can be calculated.

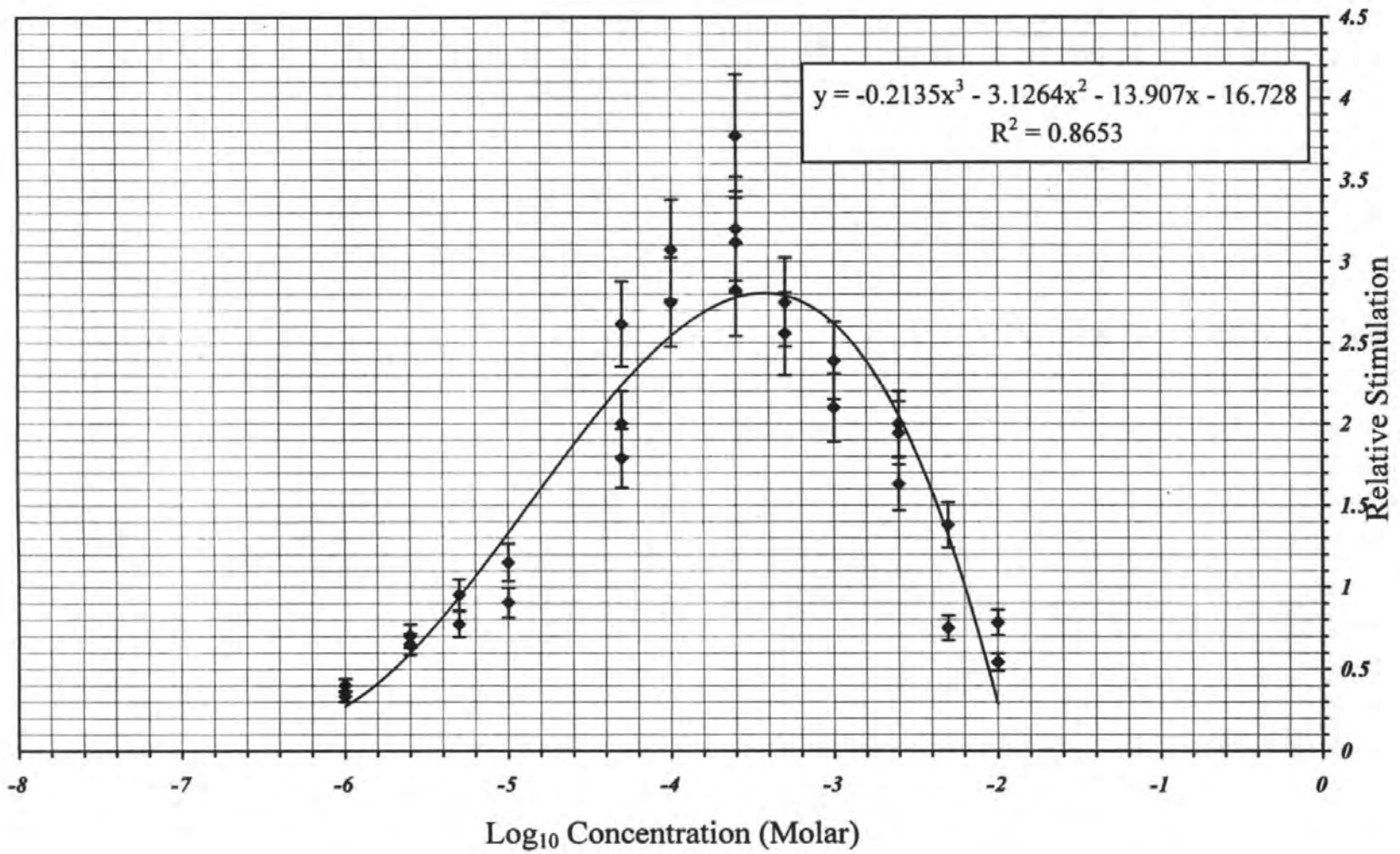


FIGURE 32 **PLOT OF BIOASSAY DATA AND REGRESSION LINE FOR PROP-2-ENYLGLUCOSINOLATE**

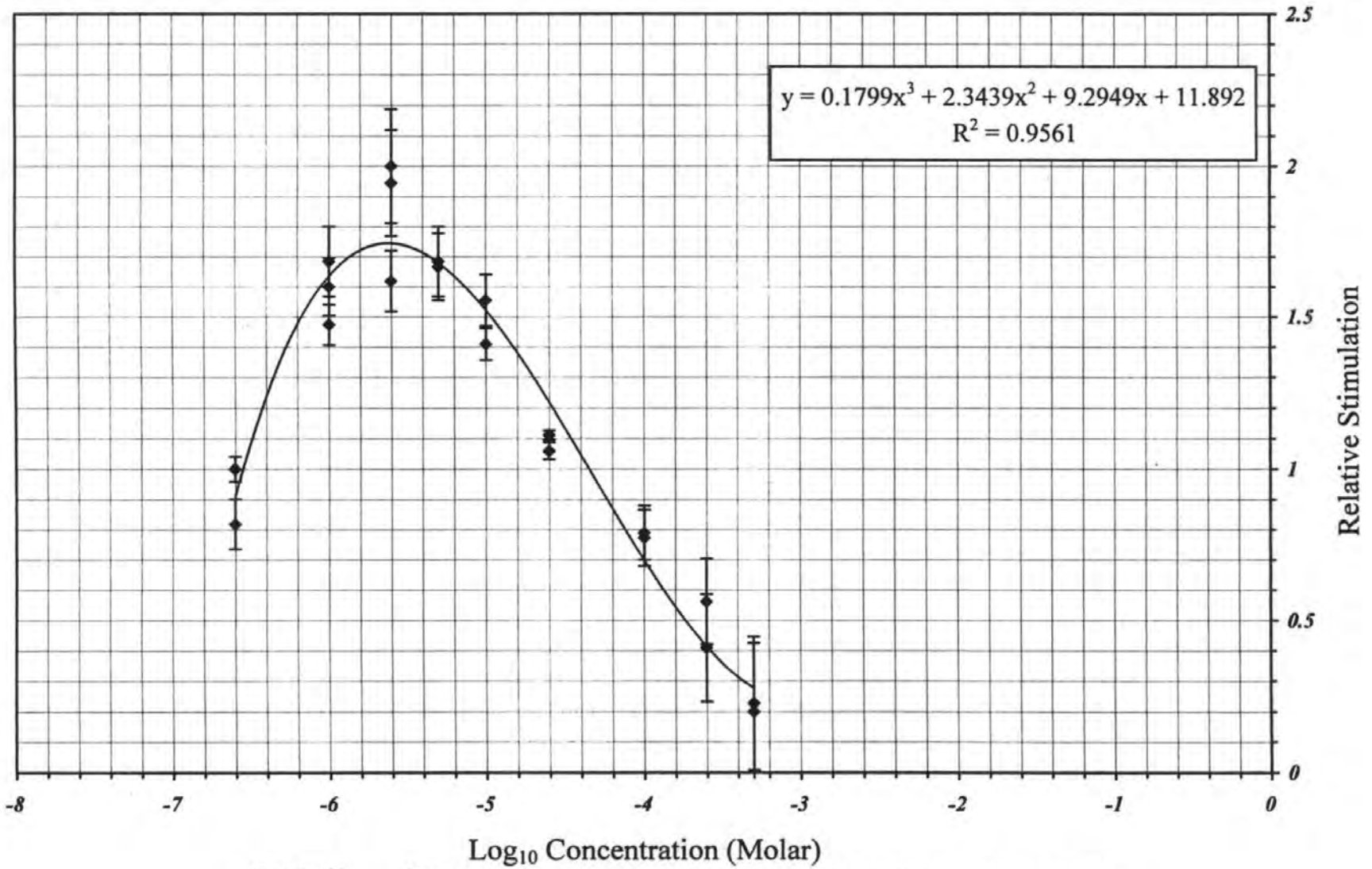


FIGURE 33 **PLOT OF BIOASSAY DATA AND REGRESSION LINE FOR BENZYLGLUCOSINOLATE**

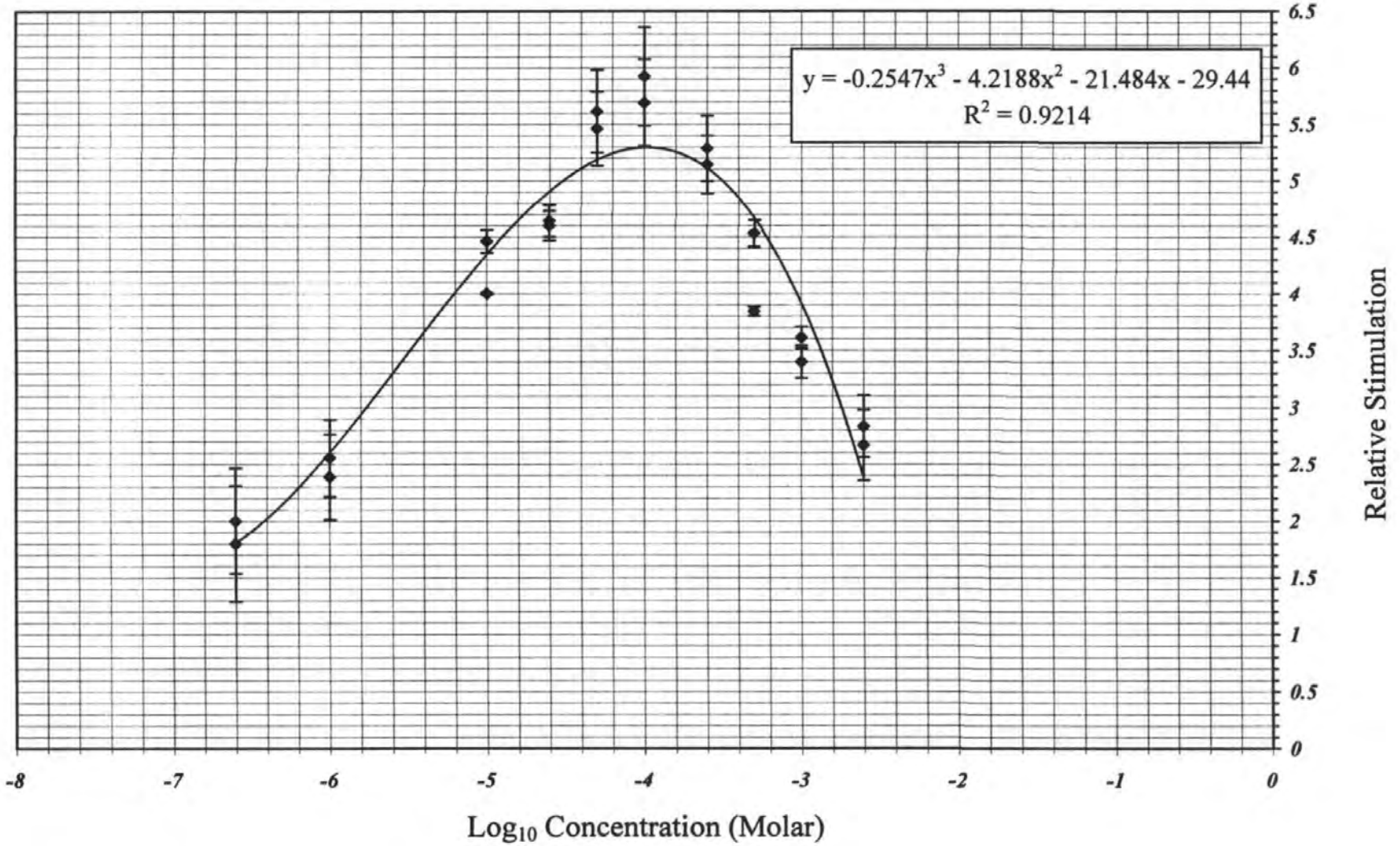


FIGURE 34 **PLOT OF BIOASSAY DATA AND REGRESSION LINE FOR INDOL-3-YL METHYLGLUCOSINOLATE**

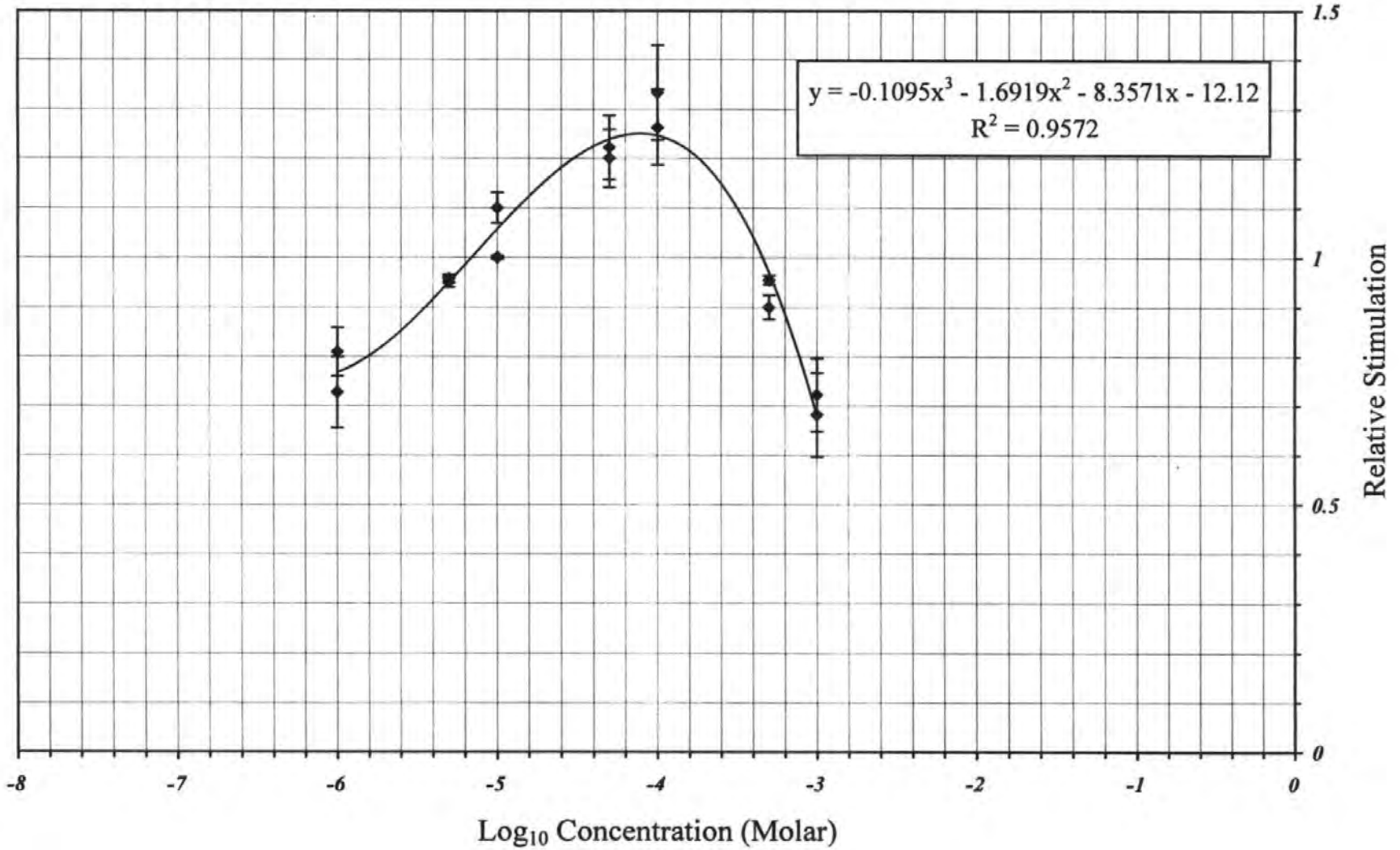


FIGURE 35 **PLOT OF BIOASSAY DATA AND REGRESSION LINE FOR POTASSIUM *2,3,4,6*-TETRA-*O*-ACETYLPROP-*2*-ENYLGLUCOSINOLATE**

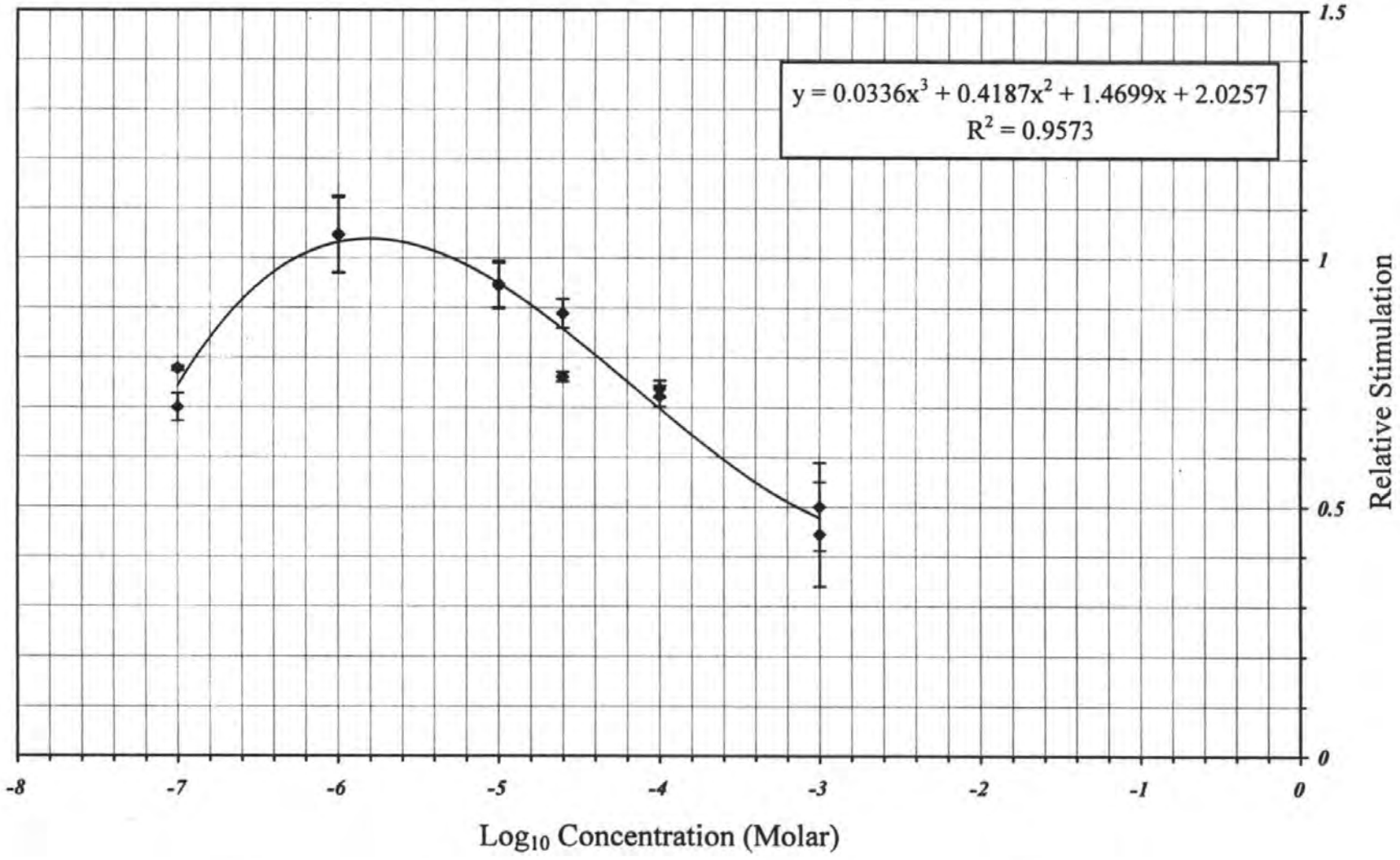


FIGURE 36 **PLOT OF BIOASSAY DATA AND REGRESSION LINE FOR *S*-METHYL CYSTEINE SULPHOXIDE**

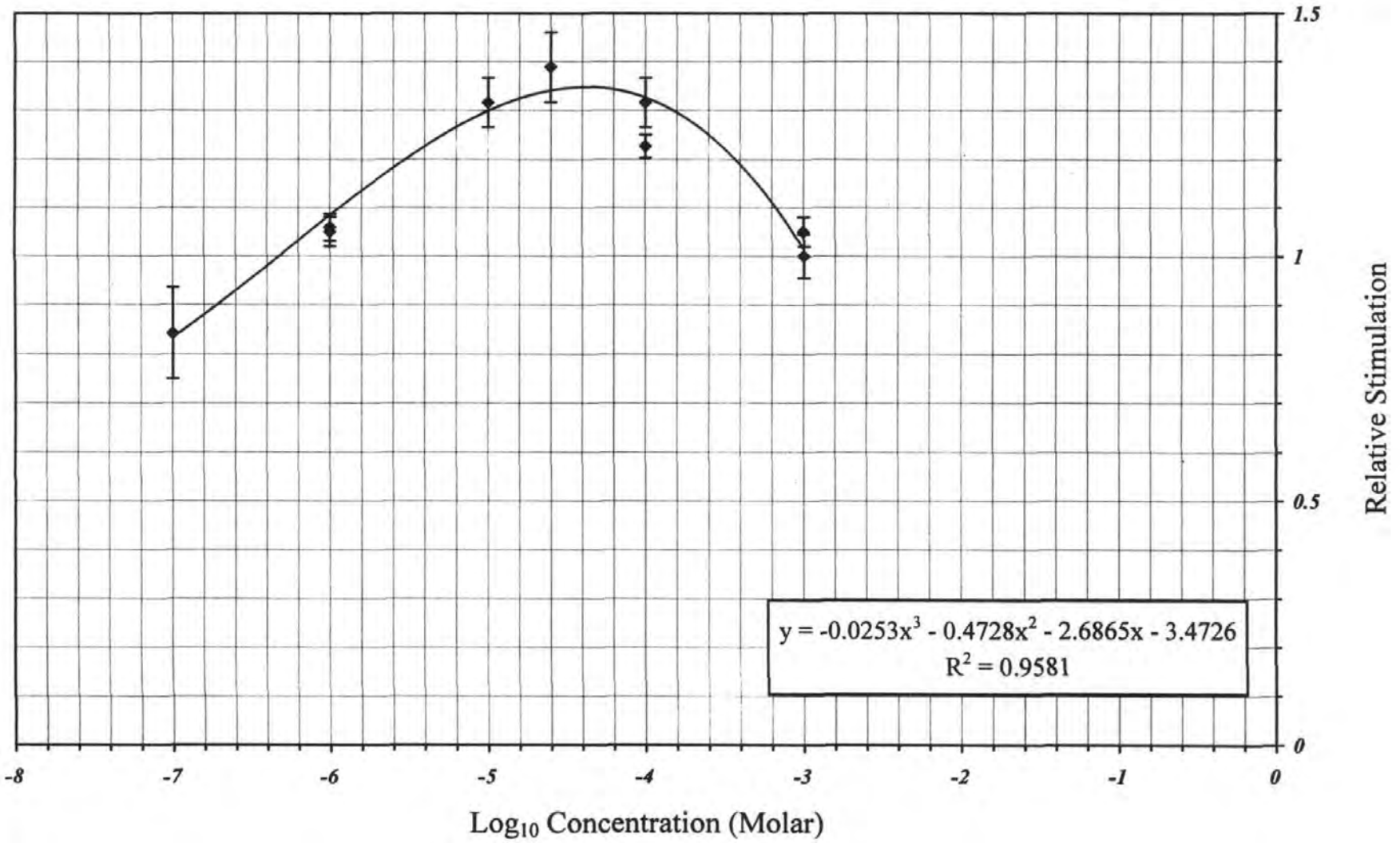


FIGURE 37 PLOT OF BIOASSAY DATA AND REGRESSION LINE FOR *S*-METHYL CYSTEINE SULPHONE

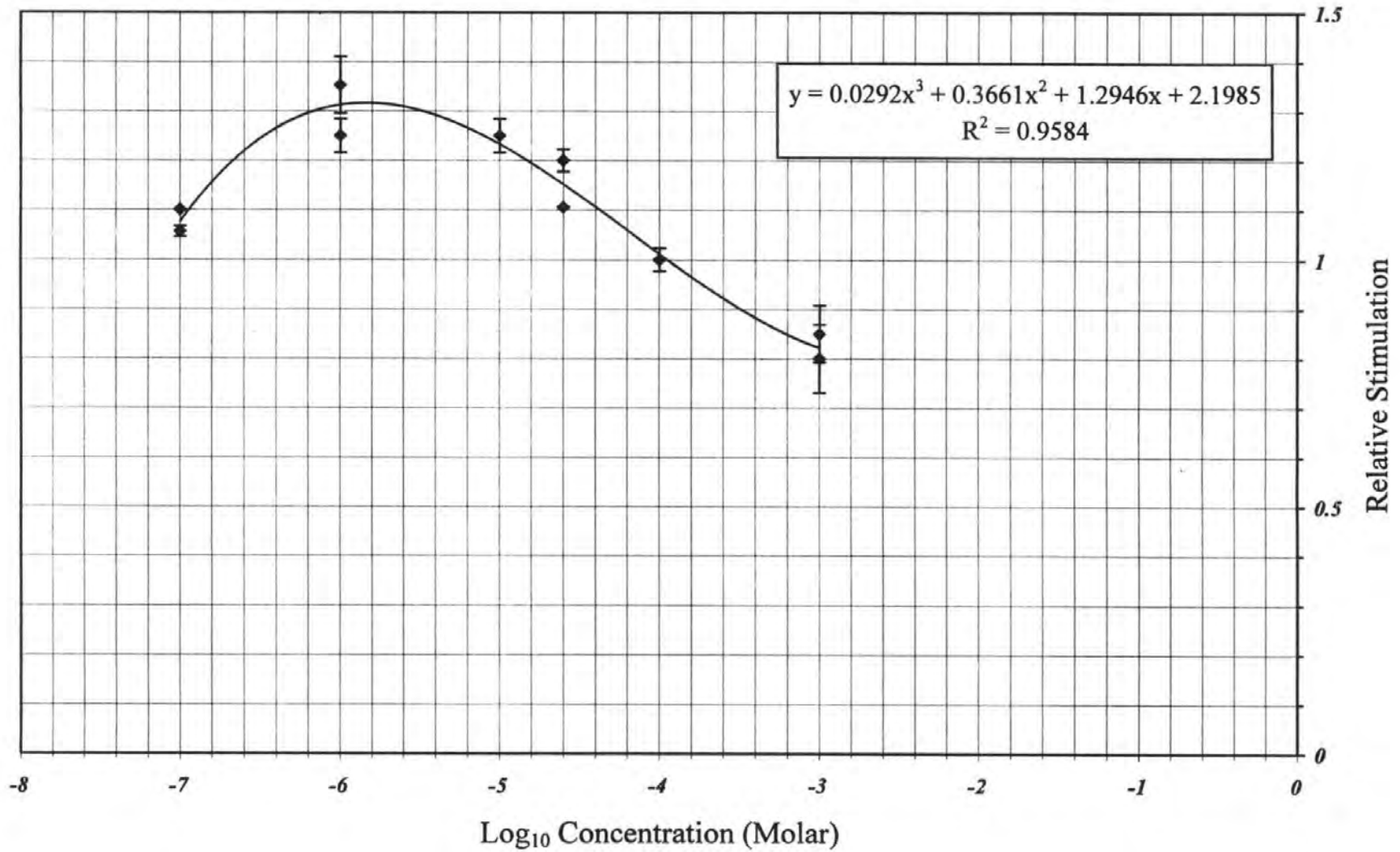


FIGURE 38 **PLOT OF BIOASSAY DATA AND REGRESSION LINE FOR CYSTEINE SULPHINIC ACID**

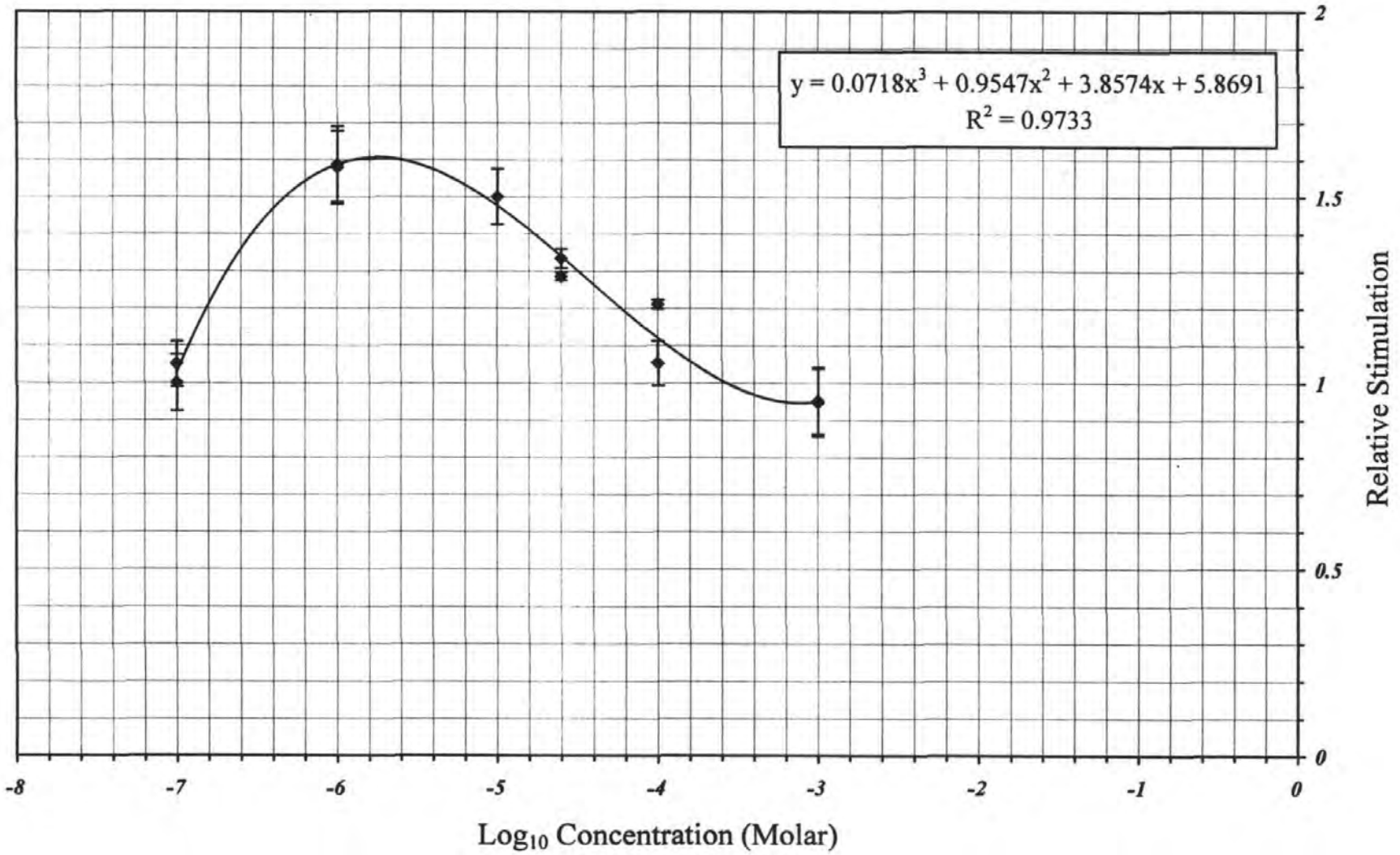


FIGURE 39 PLOT OF BIOASSAY DATA AND REGRESSION LINE FOR CYSTEIC ACID

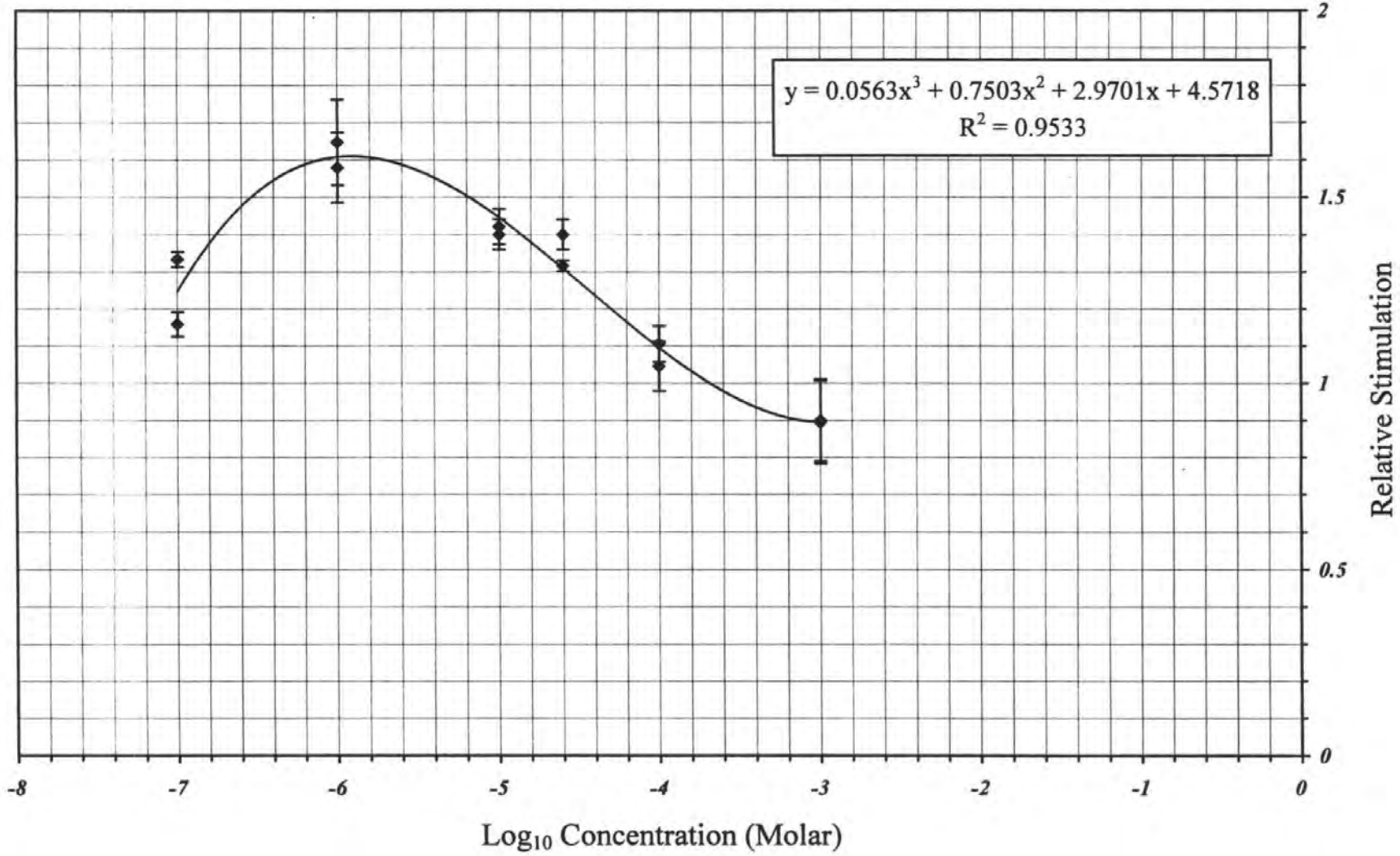


FIGURE 40 **PLOT OF BIOASSAY DATA AND REGRESSION LINE FOR TAURINE**

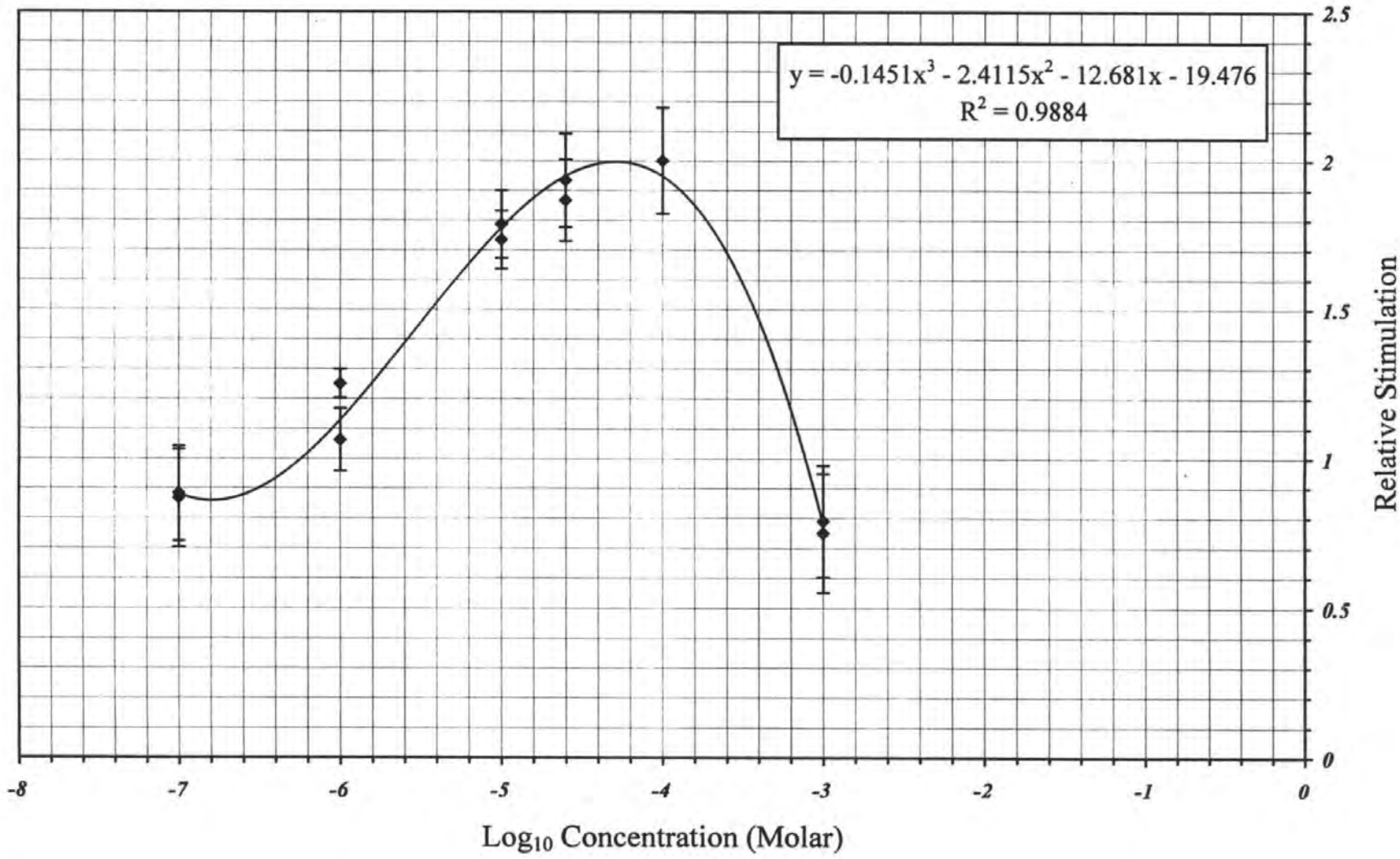


FIGURE 41 **PLOT OF BIOASSAY DATA AND REGRESSION LINE FOR SULPHAMIC ACID**

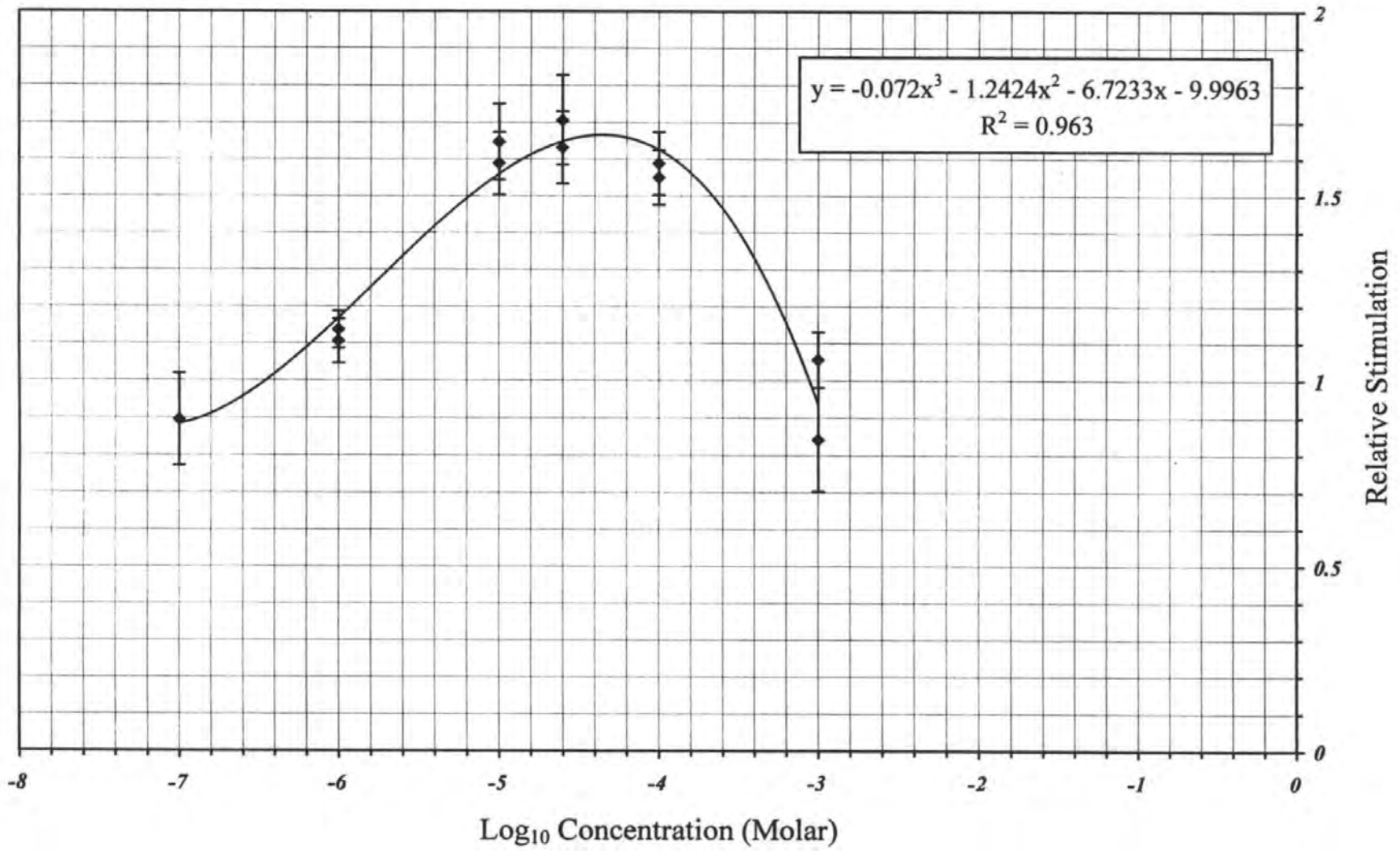


FIGURE 42 PLOT OF BIOASSAY DATA AND REGRESSION LINE FOR HYDROXYLAMINE-*O*-SULPHONIC ACID

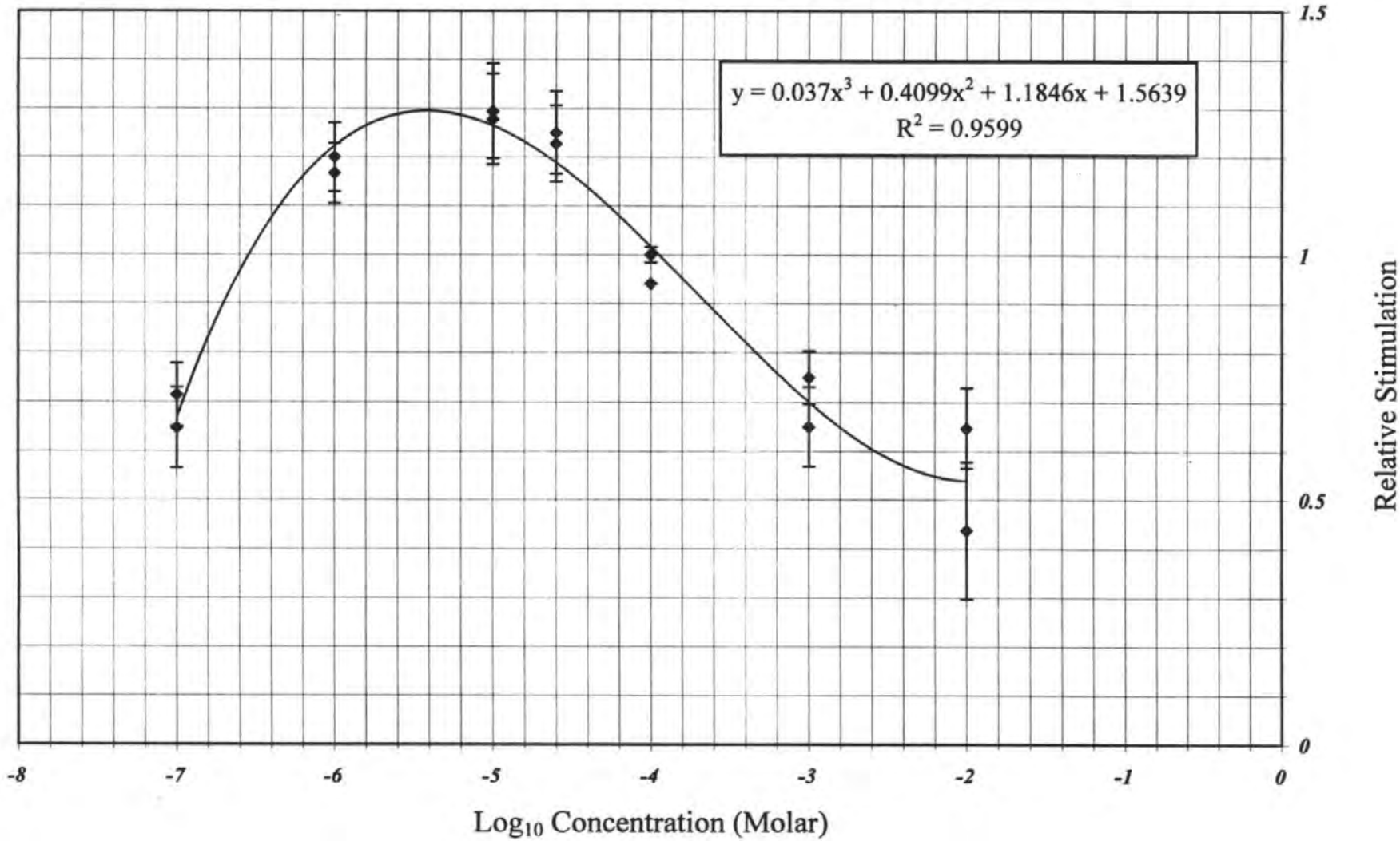


FIGURE 43 PLOT OF BIOASSAY DATA AND REGRESSION LINE FOR OCTANE SULPHONIC ACID

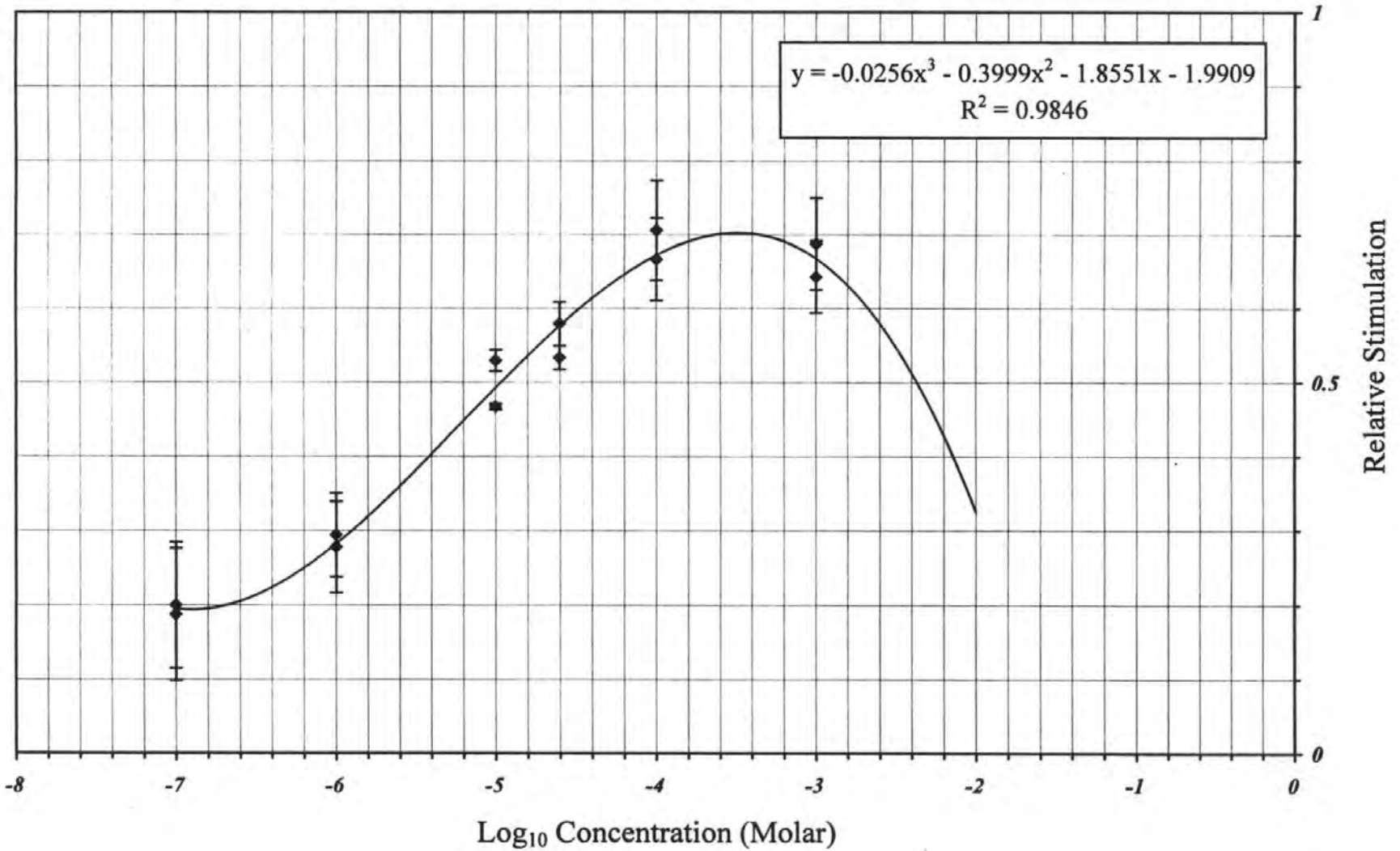


FIGURE 44 PLOT OF BIOASSAY DATA AND REGRESSION LINE FOR ACETONE SODIUM BISULPHITE

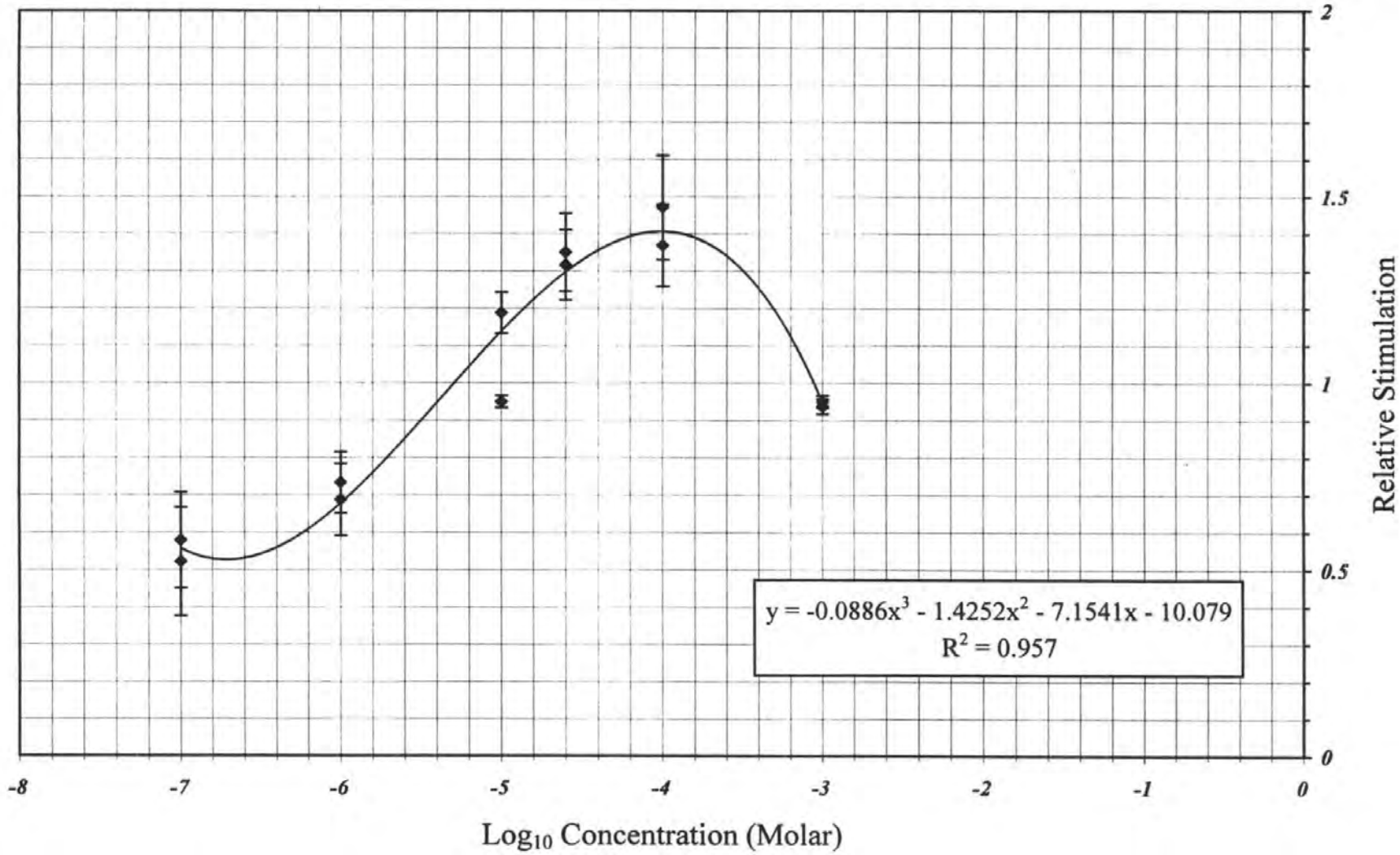


FIGURE 45 **PLOT OF BIOASSAY DATA AND REGRESSION LINE FOR *1,3,2λ-6*-BENZODIOXATHIOLE-*2,2*-DIONE**

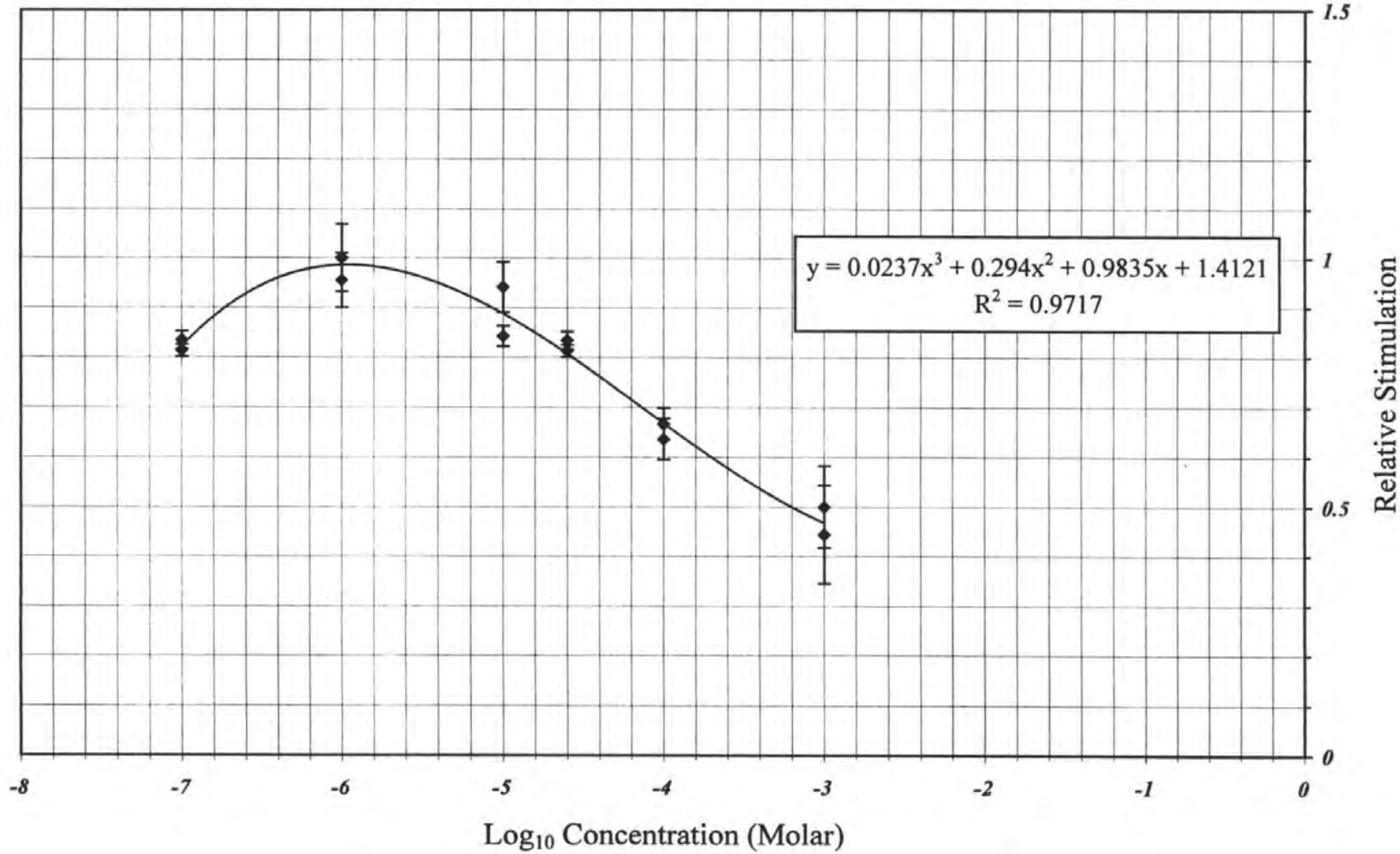


FIGURE 46 **PLOT OF BIOASSAY DATA AND REGRESSION LINE FOR *5*-NITRO-*2,3*-DIHYDRO-*1,2λ-6*-BENZOXATHIOLE-*2,2*-DIONE**

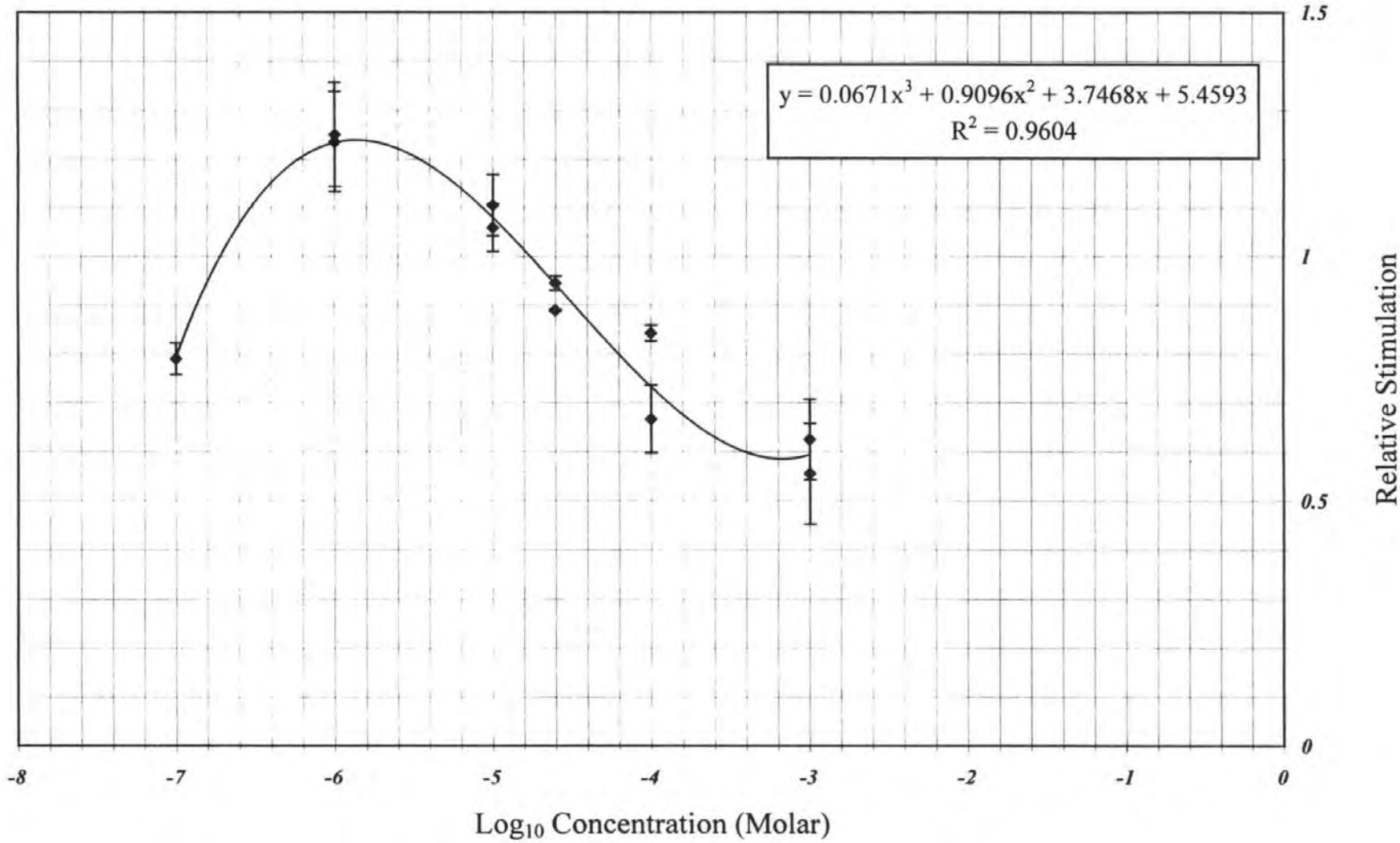


FIGURE 47 PLOT OF BIOASSAY DATA AND REGRESSION LINE FOR TOLUENE -*P*-SULPHONAMIDE

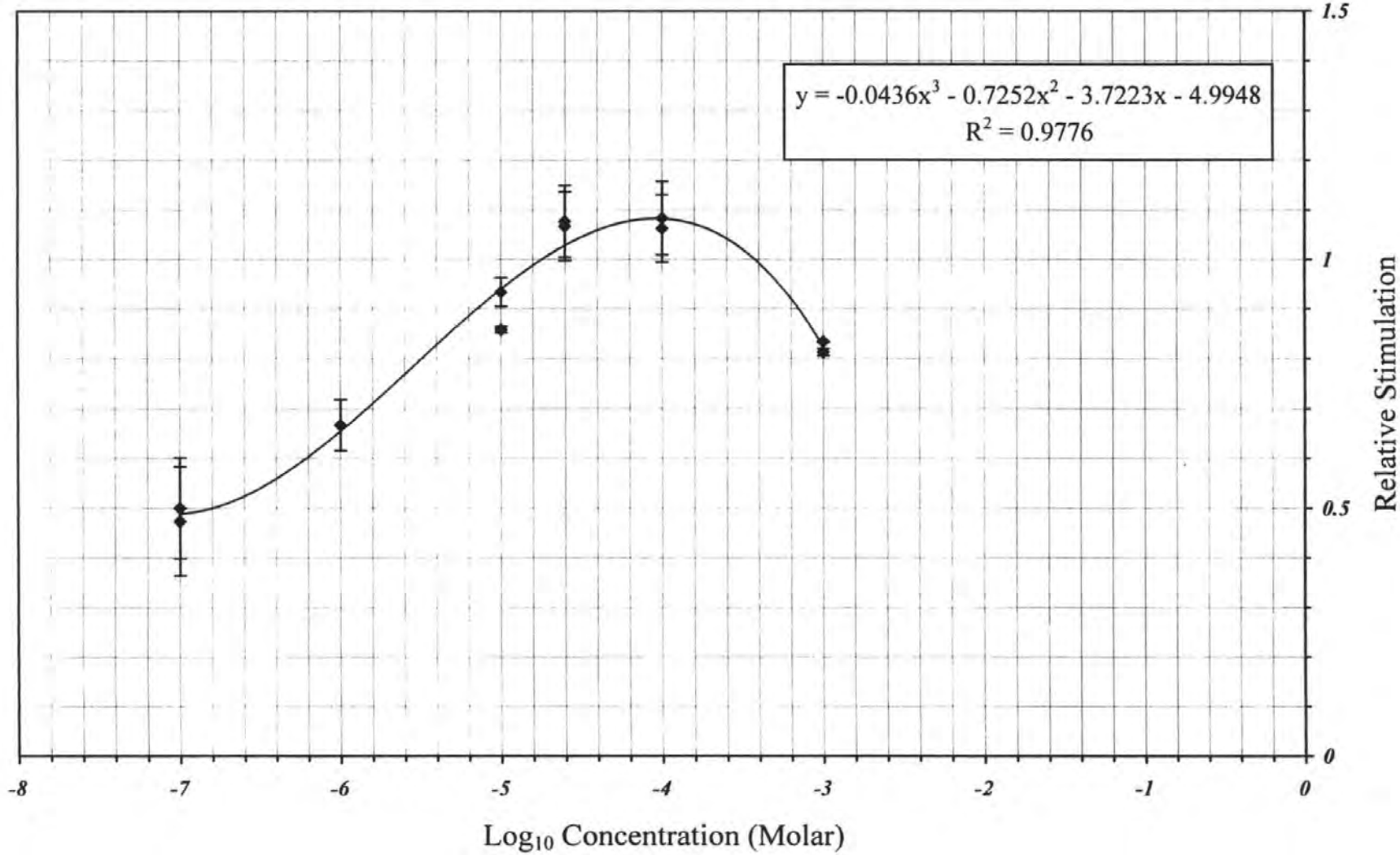


FIGURE 48 **PLOT OF BIOASSAY DATA AND REGRESSION LINE FOR TOLUENE-*P*-SULPHONIC ACID**

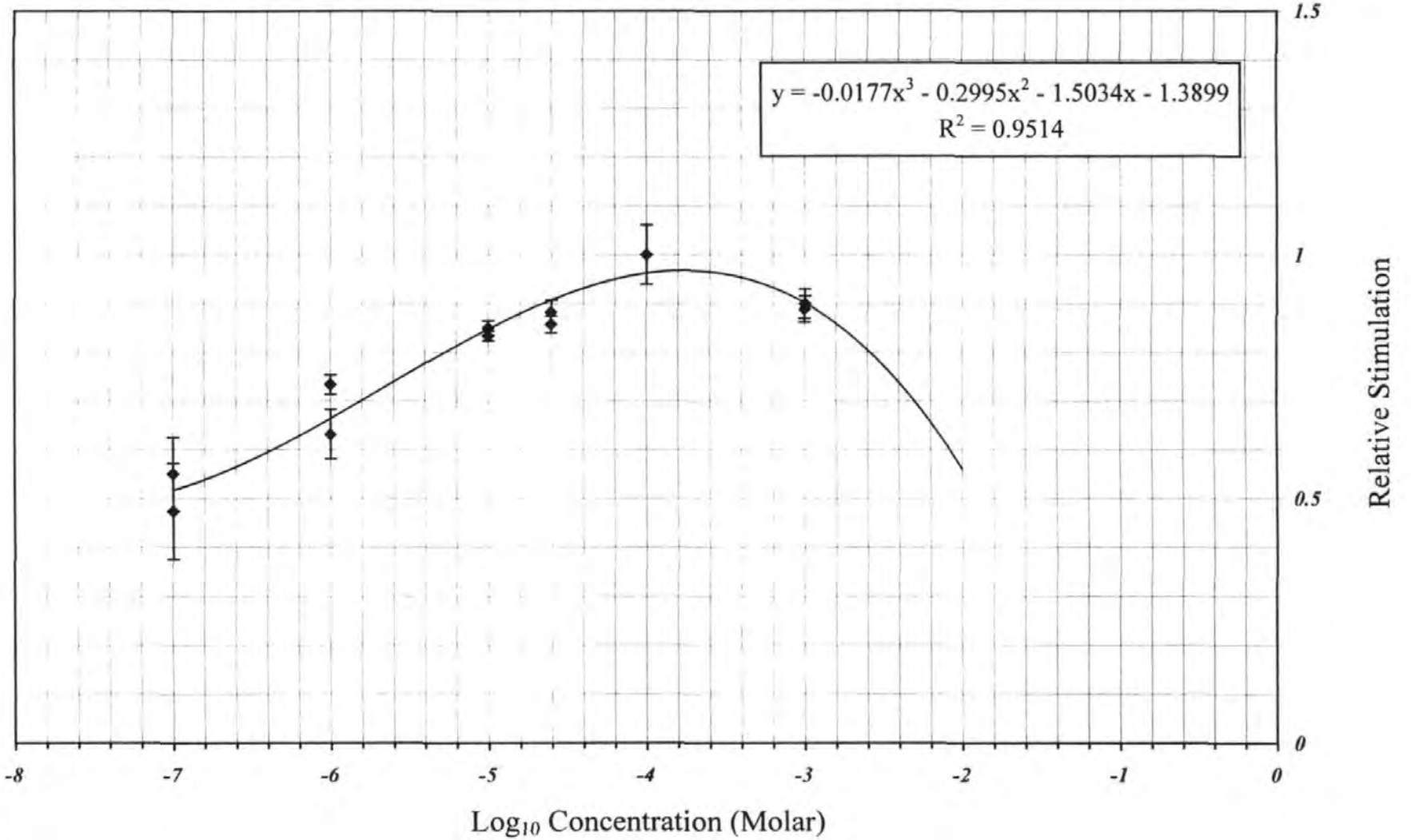


FIGURE 49 **PLOT OF BIOASSAY DATA AND REGRESSION LINE FOR POTASSIUM TOLUNE-*P*-SULPHONATE**

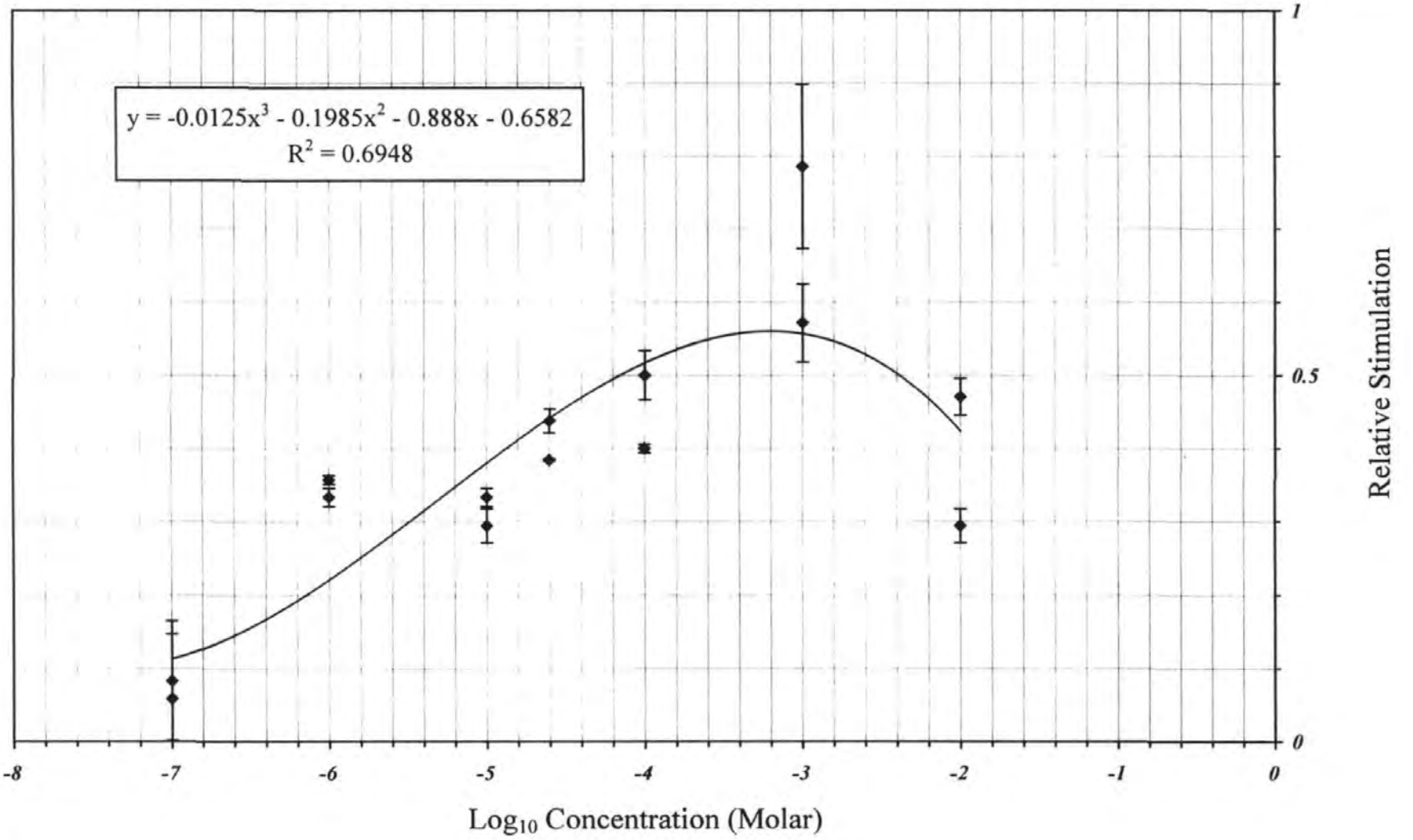


FIGURE 50 **PLOT OF BIOASSAY DATA AND REGRESSION LINE FOR GRAMINE TOLUENE-*P*-SULPHONATE**

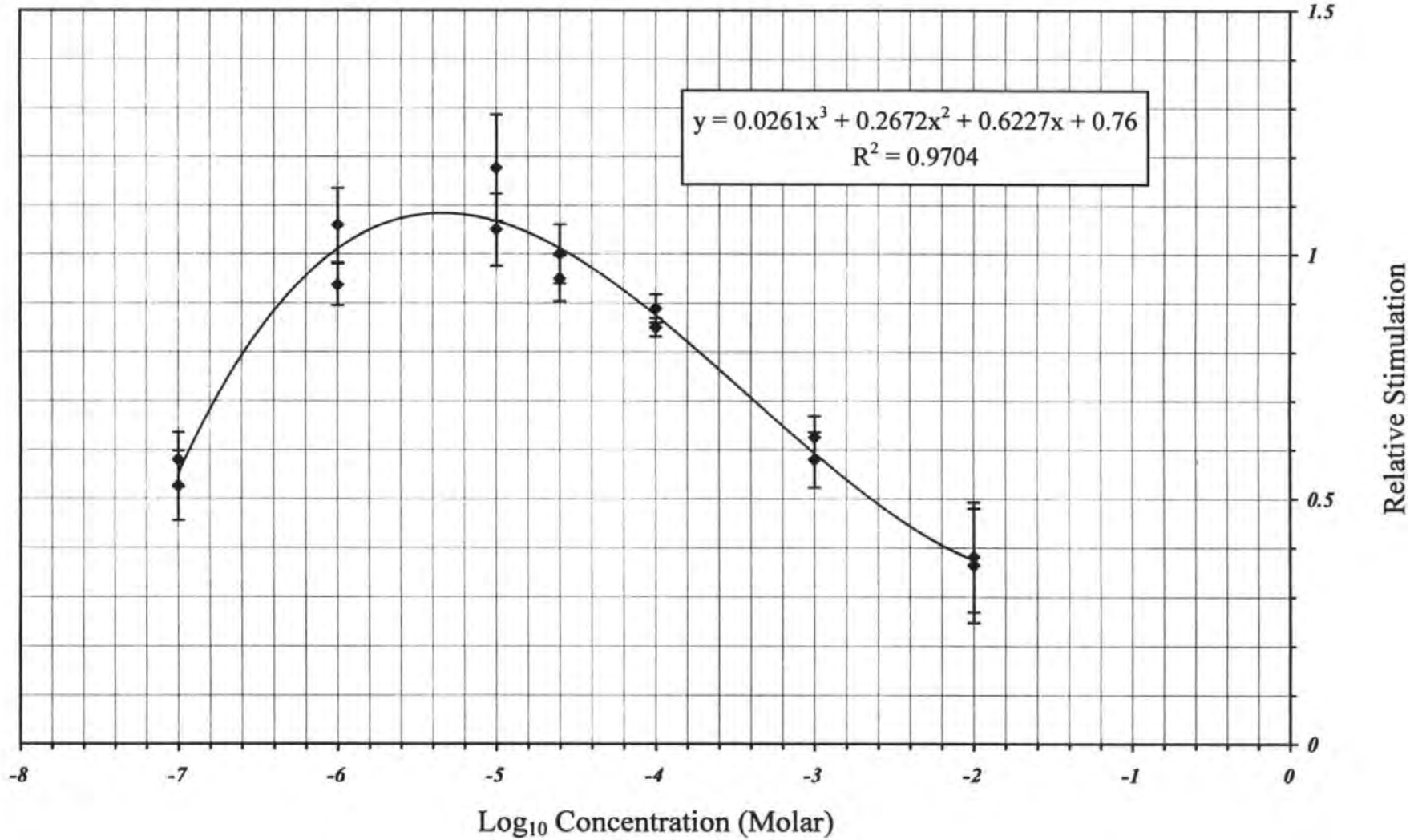


FIGURE 51 **PLOT OF BIOASSAY DATA AND REGRESSION LINE FOR INDOLE-3-ACETALDEHYDE SODIUM BISULPHITE**

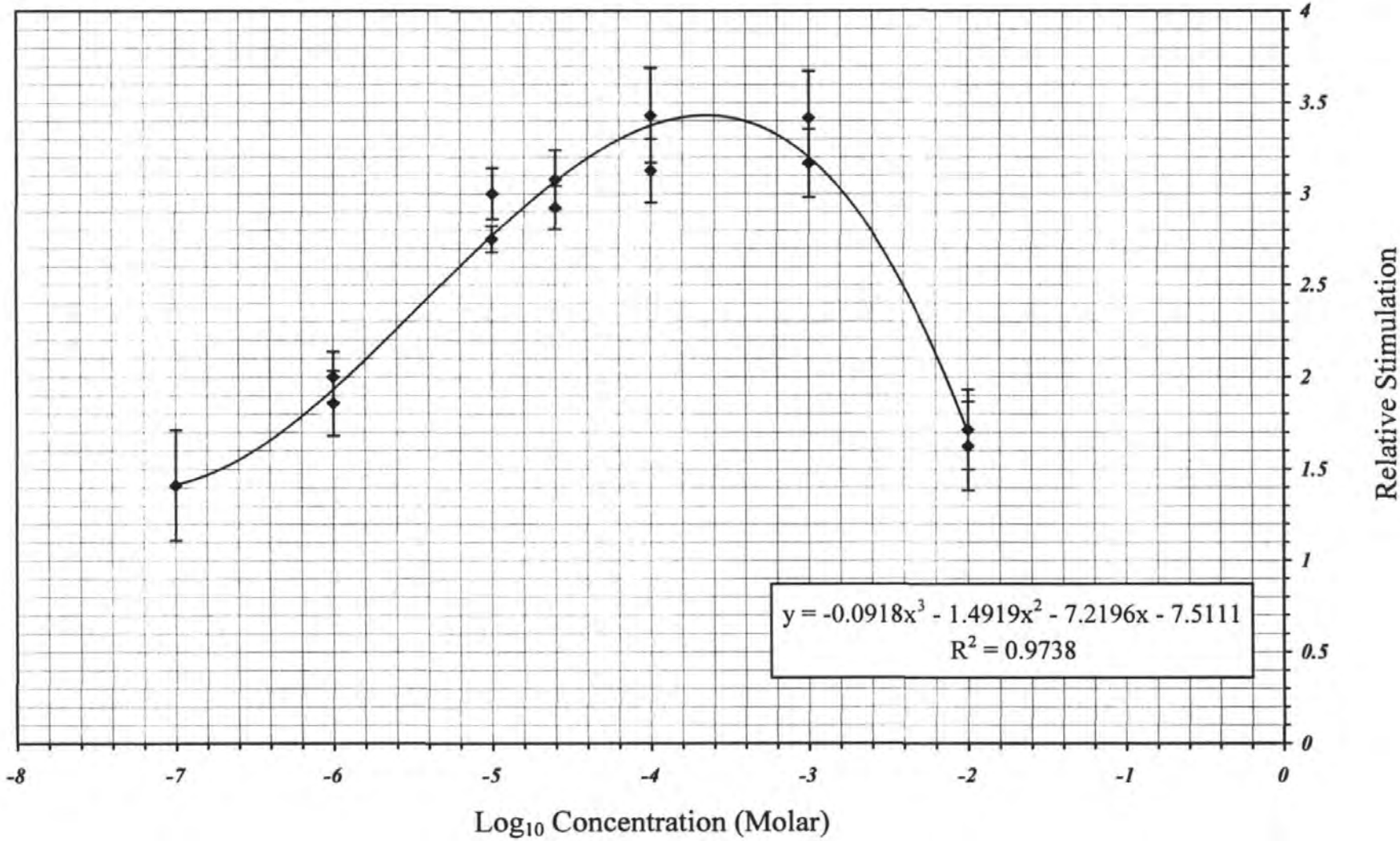


FIGURE 52 **PLOT OF BIOASSAY DATA AND REGRESSION LINE FOR INDOXYL-*3*-SULPHATE, POTASSIUM SALT**

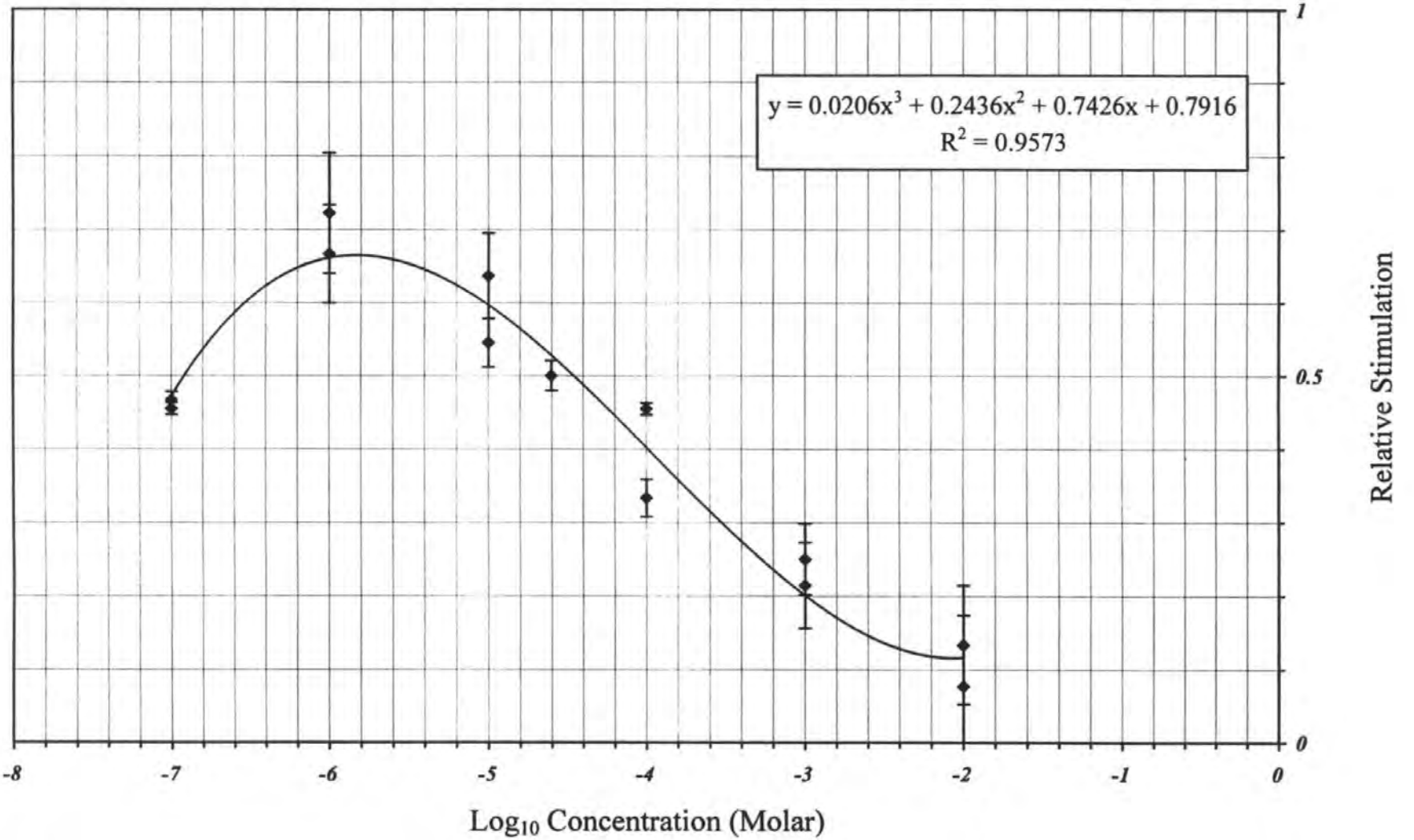


FIGURE 53 **PLOT OF BIOASSAY DATA AND REGRESSION LINE FOR *1*-TOLUENE-*4*-SULPHONYL)-INDOLE**

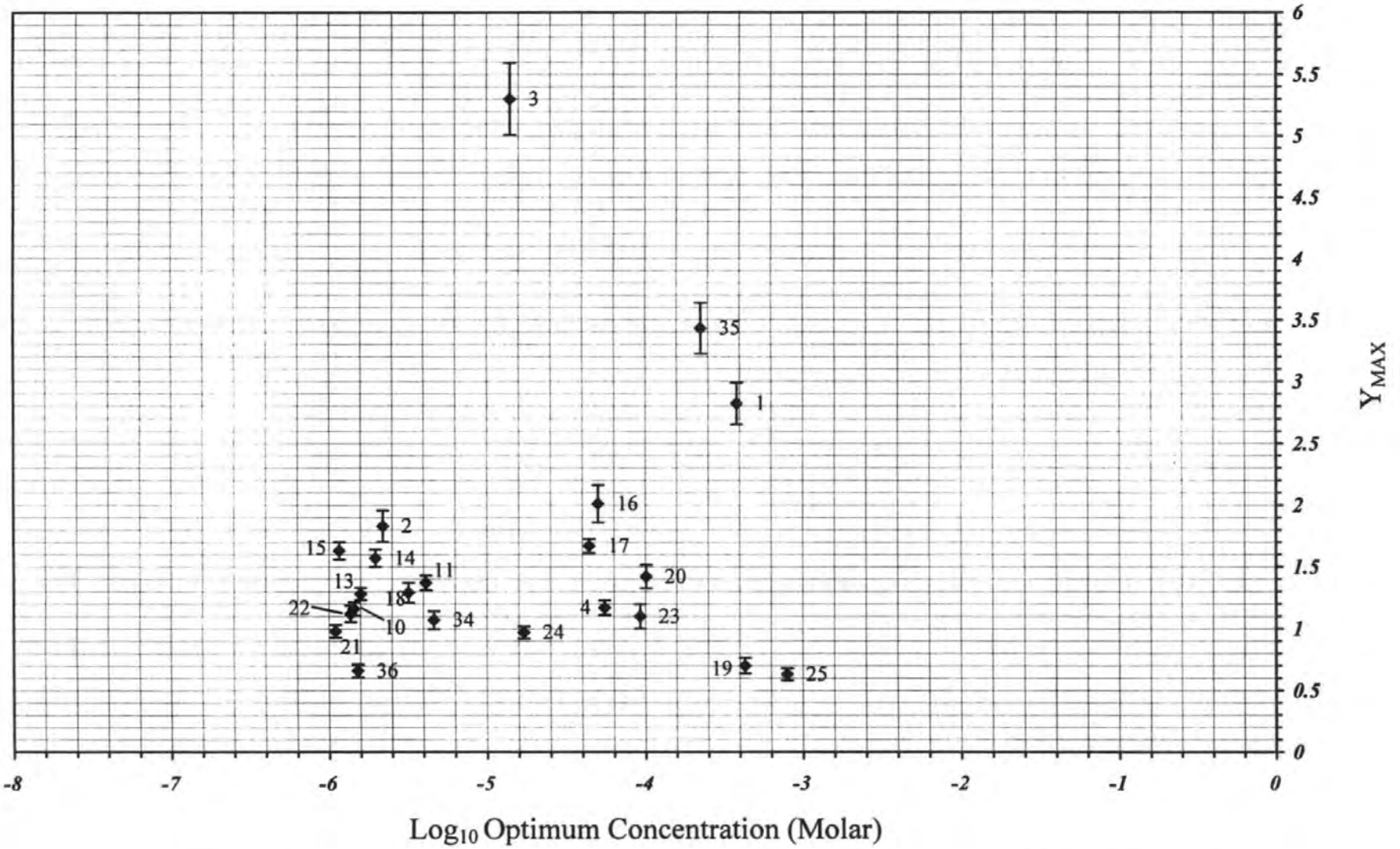


FIGURE 54 **PLOT OF OPTIMUM CONCENTRATIONS AND OPTIMUM RESPONSES FOR ALL ACTIVE COMPOUNDS**

It is acknowledged that there are certain limitations to the generation of the "best fit curves". The Microsoft Excel 5 software creates an empirical model and not a theoretical best fit. Examination of the graphs shows that for the majority of the compounds tested the calculated optimum responses, Y_{MAX} appears to be an underestimate of the actual optimum responses. However for all the active compounds at (P=5%) there is a significant positive correlation between the relative oviposition response and test concentration as represented by the best fit curve which therefore provides a statistically sound model of the data. Work by Billington (1991) on isothiocyanates as attracants for *D. radicum* larvae produced data plots of $\log_{10}$ concentration against percentage response which could be modelled using a quadratic equation giving a parabolic curve of best fit. The results recorded by Billington (1991) showed true repellancy at the higher test solution concentrations. In this study the number or eggs actually laid was never less than the number laid on the blank surrogate leaves and so true repellancy was not observed, only a decrease in stimulatory activity at the higher concentrations.

Figure 54 shows a plot of maximum relative stimulation (Y_{MAX}) ± standard error against optimum concentration (C) for each compound. With the exception of prop-*2*-enyl glucosinolate (1), indol-3-yl methylglucosinolate (3) and indoxyl-*3*-sulphate, potassium salt (35) all the bioactive compounds lie within a narrow range of Y_{MAX} values.

Oviposition stimulation as the efficacy of compounds can be assessed in two ways. Firstly, the lower the optimum concentration (C) the greater the biological activity for a compound. Secondly, the higher the value of Y_{MAX} for a compound the greater its stimulatory activity. Table 7 shows the active test compounds in decreasing biological activity.

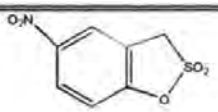
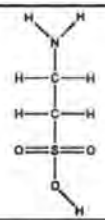
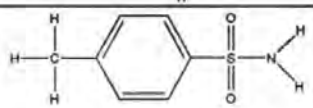
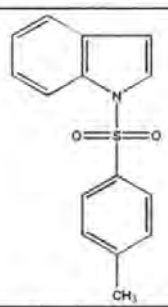
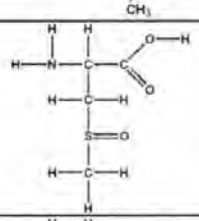
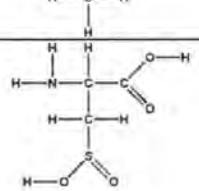
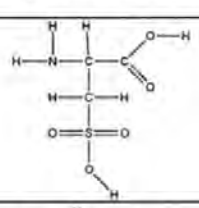
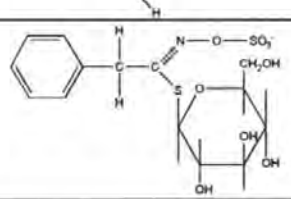
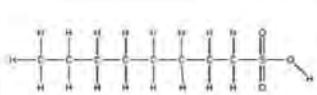
No.	Structure	Compound Name	C (Molar)	Y_{MAX} ± Std Error
21		*5*-Nitro-*2,3*-dihydro-*1,2λ-6*-benzoxathiole-*2,2*-dione	1.10×10^{-6}	0.98 ± 0.06
15		Taurine	1.16×10^{-6}	1.63 ± 0.07
22		Toluene-*p*-sulphonamide	1.37×10^{-6}	1.12 ± 0.06
36		*1*-(Toluene-*4*-sulphonyl)-indole	1.52×10^{-6}	0.66 ± 0.05
10		*S*-Methyl cysteine sulphoxide	1.44×10^{-6}	1.16 ± 0.06
13		Cysteine sulphinic acid	1.59×10^{-6}	1.28 ± 0.05
14		Cysteic acid	1.97×10^{-6}	1.57 ± 0.07
2		Benzylglucosinolate	2.2×10^{-6}	1.83 ± 0.13
18		Octane sulphonic acid	3.19×10^{-6}	1.29 ± 0.08

Table 7 **Names, Structure and Details of Test Compounds in Decreasing Biological Activity (C)**

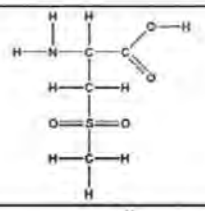
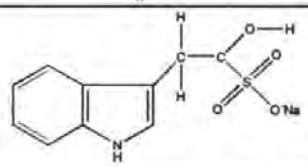
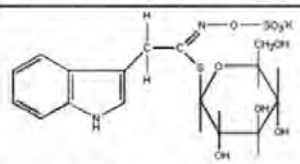
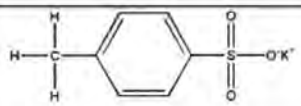
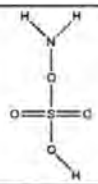
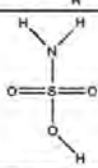
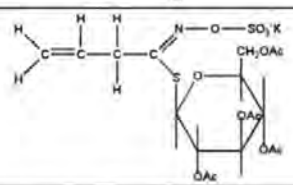
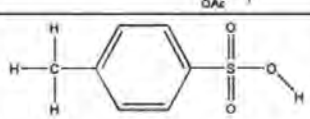
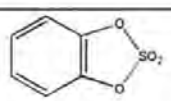
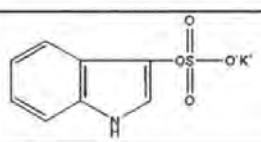
11		*S*-Methyl cysteine sulphone	4.11×10^{-6}	1.37 ± 0.06
34		Indole acetaldehyde sodium bisulphite	4.63×10^{-6}	1.07 ± 0.07
3		Indol-*3*-yl methyl glucosinolate	1.4×10^{-5}	5.30 ± 0.29
24		Potassium toluene-*p*-sulphonate	1.70×10^{-5}	0.97 ± 0.05
17		Hydroxylamine-*O*-sulphonic acid	4.39×10^{-5}	1.67 ± 0.06
16		Sulphamic acid	4.99×10^{-5}	2.01 ± 0.15
4		Potassium *2,3,4,6*-tetra-*O*-acetyl prop-*2*-enylglucosinolate	5.5×10^{-5}	1.17 ±0.06
23		Toluene -*p*-sulphonic acid	9.25×10^{-5}	1.10 ± 0.1
20		*1,3,2λ-6*-Benzodioxathiole-*2,2*-dione	1.01×10^{-4}	1.42 ± 0.1
35		Indoxyl-*3*-sulphate, potassium salt	2.25×10^{-4}	3.43 ± 0.2

TABLE 7 CONTINUED

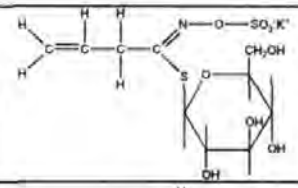
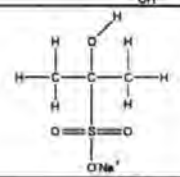
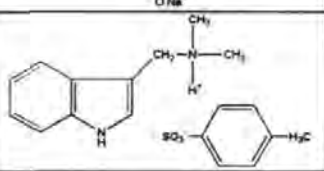
1		Prop-2-enylglucosinolate	3.8×10^{-4}	2.82 ± 0.16
19		Acetone sodium bisulphite	4.29×10^{-4}	0.70 ± 0.06
25		Gramine toluene-*p*-sulphonate	7.94×10^{-4}	0.63 ± 0.05

TABLE 7 CONTINUED

Table 8 shows the results of the active test compounds in decreasing order of stimulatory (Y_{MAX}) activity. This index of activity is related to the total number of eggs laid on the surrogate leaves.

In the oviposition studies reported by Städler and Roessingh (1990), Roessingh *et al.*, (1992), Simmonds *et al.*, (1994), Hopkins *et al.*, (1997) and Roessingh *et al.*, (1997) the efficacy of a compound as an oviposition stimulant was determined soley by the maximum number of eggs laid from a range of test concentrations. The significance, if any, of the optimum concentration of a test compound (C) was not discussed.

In contrast to the optimum concentration, the total number of eggs laid on the test sites and, therefore Y_{MAX} is dependant on the bioassay conditions. A gravid female fly is only able to lay a finite but variable number of eggs. Under laboratory conditions a gravid female cabbage root fly is reported to lay between 9 and 12 eggs in a 24 hour period (Smithers, pers. Comm.). Work by Braven *et al.*, (unpublished) suggests that a total of 30 or more eggs laid on a single oviposition site would begin to deter further oviposition at that site by other gravid females. As describeed in section *2.1.2* the bioassay procedure used in this study was designed to minimise any oviposition deterrence effects even if there was maximum egg deposition by all the insects during the experiment.

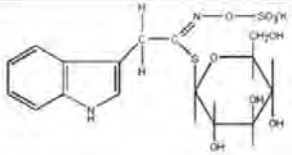
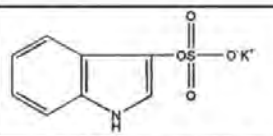
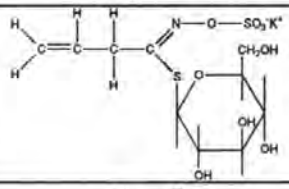
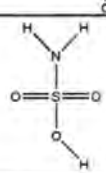
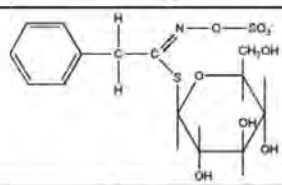
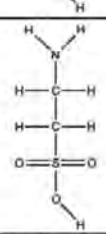
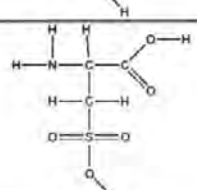
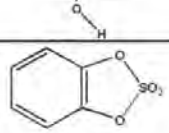
No.	Structure	Compound Name	C (Molar)	Y_{MAX} ± Std Error
3		Indol-*3*-yl methyl glucosinolate	1.4×10^{-5}	5.30 ± 0.29
35		Indoxyl-*3*-sulphate, potassium salt	2.25×10^{-4}	3.43 ± 0.2
1		Prop-*2*-enylglucosinolate	3.8×10^{-4}	2.82 ± 0.16
16		Sulphamic acid	4.99×10^{-5}	2.01 ± 0.15
2		Benzylglucosinolate	2.2×10^{-6}	1.83 ± 0.13
17		Hydroxylamine-*O*-sulphonic acid	4.39×10^{-5}	1.67 ± 0.06
15		Taurine	1.16×10^{-6}	1.63 ± 0.07
14		Cysteic acid	1.97×10^{-6}	1.57 ± 0.07
20		*1,3,2λ-6*-Benzodioxathiole-*2,2*-dione	1.01×10^{-4}	1.42 ± 0.1

Table 8 **Names, Structures and Details of Test Compounds in Decreasing Stimulatory Activity**

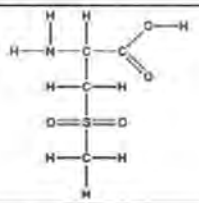
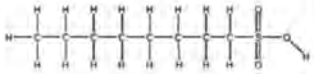
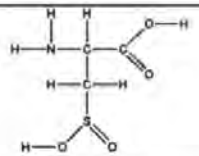
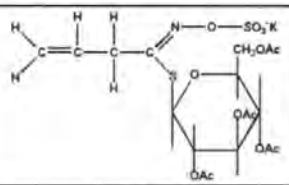
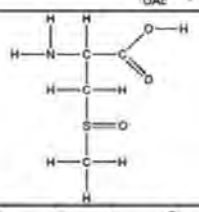
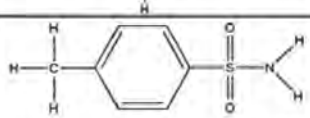
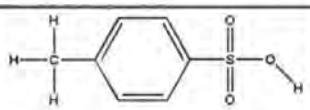
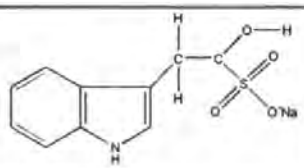
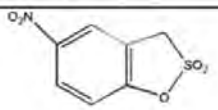
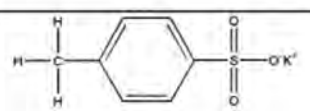
11		*S*-Methyl cysteine sulphone	4.11×10^{-6}	1.37 ± 0.06
18		Octane sulphonic acid	3.19×10^{-6}	1.29 ± 0.08
13		Cysteine sulphinic acid	1.59×10^{-6}	1.28 ± 0.05
4		Potassium *2,3,4,6*-tetra-*O*-acetyl prop-*2*-enylglucosinolate	5.5×10^{-5}	1.17 ±0.06
10		*S*-Methyl cysteine sulphoxide	1.44×10^{-6}	1.16 ± 0.06
22		Toluene-*p*-sulphonamide	1.37×10^{-6}	1.12 ± 0.06
23		Toluene -*p*-sulphonic acid	9.25×10^{-5}	1.10 ± 0.1
34		Indole-*3*-acetaldehyde sodium bisulphite	4.63×10^{-6}	1.07 ± 0.07
21		*5*-Nitro-*2,3*-dihydro-*1,2λ-6*-benzoxathiole-*2,2*-dione	1.10×10^{-6}	0.98 ± 0.06
24		Potassium toluene-*p*-sulphonate	1.70×10^{-5}	0.97 ± 0.05

TABLE 8 CONTINUED

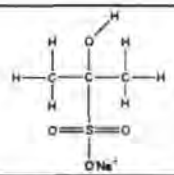
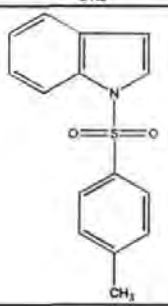
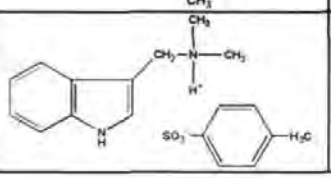
19		Acetone sodium bisulphite	4.29×10^{-4}	0.70 ± 0.06
36		*1*-(Toluene-*4*-sulphonyl)-indole	1.52×10^{-6}	0.66 ± 0.05
25		Gramine toluene-*p*-sulphonate	7.94×10^{-4}	0.63 ± 0.05

TABLE 8 CONTINUED

A potent oviposition stimulant would have a high Y_{MAX} and a low optimum concentration. One way to assess this is to calculate a ratio of stimulatory activity and biological activity, i.e. Y_{MAX} / C. Table 9 shows the active test compounds in decreasing ratio. It can be seen from Table 9 that Taurine (15) has the largest Y_{MAX} / C value and therefore has the best compromise between optimum relative response and optimum concentration (Y_{MAX} = 1.63 ± 0.07, C = 1.16×10^{-6} M). On this basis it would be the best chemical to select for practical purposes.

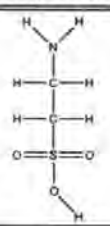
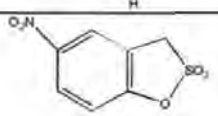
NO.	STRUCTURE	COMPOUND NAME	Y_{MAX} / C
15		Taurine	1405172
21		*5*-Nitro-*2,3*-dihydro-*1,2λ-6*-benzoxathiole-*2,2*-dione	890909

TABLE 9 NAMES, STRUCTURES AND DETAILS OF TEST COMPOUNDS IN DECREASING Y_{MAX} / C RATIO ORDER

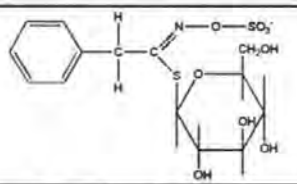
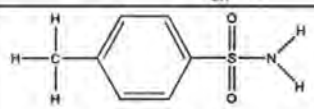
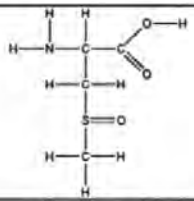
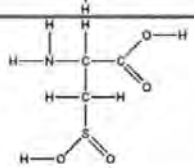
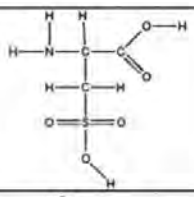
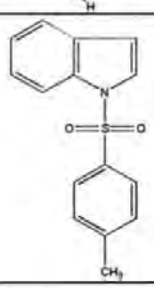
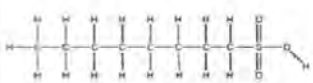
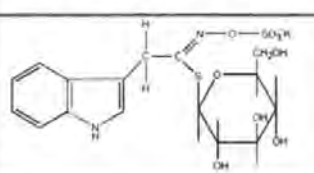
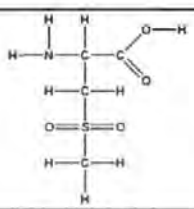
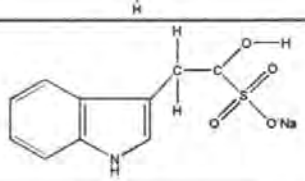
2		Benzylglucosinolate	831818
22		Toluene-*p*-sulphonamide	817518
10		*S*-Methyl cysteine sulphoxide	805555
13		Cysteine sulphinic acid	805031
14		Cysteic acid	796954
36		*1*-(Toluene-*4*-sulphonyl)-indole	434210
18		Octane sulphonic acid	404388
3		Indol-*3*-yl methyl glucosinolate	378571
11		*S*-Methyl cysteine sulphone	333333
34		Indole-*3*-acetaldehyde sodium bisulphite	231101

TABLE 9 CONTINUED

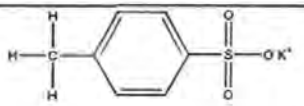
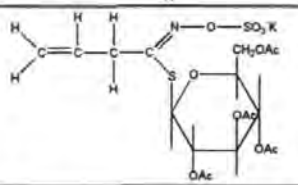
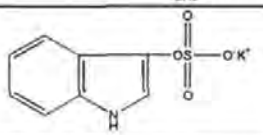
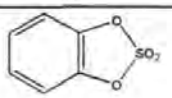
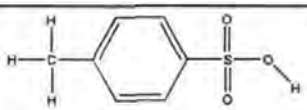
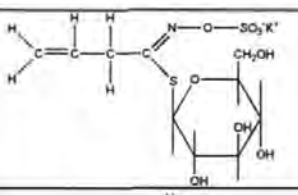
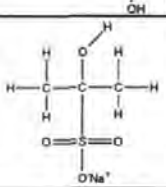
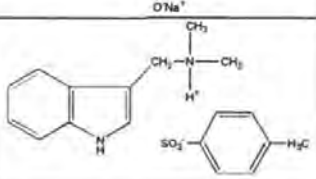
24		Potassium toluene-*p*-sulphonate	57058
16		Sulphamic acid	40280
17		Hydroxylamine-*O*-sulphonic acid	38041
4		Potassium *2,3,4,6*-tetra-*O*-acetyl prop-*2*-enylglucosinolate	21272
35		Indoxyl-*3*-sulphate, potassium salt	15244
20		*1,3,2λ-6*-benzodioxathiole-*2,2*-dione	14059
23		Toluene -*p*-sulphonic acid	11891
1		Prop-*2*-enylglucosinolate	7421
19		Acetone sodium bisulphite	1631
25		Gramine toluene-*p*-sulphonate	793

TABLE 9 CONTINUED

The naturally occurring glucosinolate molecules can be divided into three structural regions: a side-chain (R), a thioglucose and a sulphated oxime. Examination of Table 6 compounds 1 to 3 shows that variations in R result in changes of activity, demonstrated by prop-*2*-enylglucosinolate, indol-*3*-yl methylglucosinolate and benzylglucosinolate.

Städler (1978) showed that the D type sensilla on the prothoracic leg of *Delia radicum* were more responsive to prop-*2*-enylglucosinolate than type A sensilla. Roessingh and co-workers (1992) showed that the D sensilla on tarsal segments 3 and 4 (D_3 and D_4) contain sensitive receptor cells for glucosinolates. In contrast, the receptor cells of the D sensilla on the other segments did not respond in a dose dependent way to these compounds.

From their results Roessingh *et al.* (1992) concluded that the observed order of effectiveness in behavioural and electrophysiological terms corresponded well and that there was a significant correlation between the overall length of the glucosinolate's side chain and stimulatory activity. However the outstanding activity of indol-*3*-ylglucosinolate could not be correlated with the electrophysiological data. It was suggested that this could be due to the fly having additional glucosinolate receptors with a higher specificity for indol-*3*-yl methylglucosinolate.

Roessingh *et al.* (1992) found indol-*3*-yl methylglucosinolate to have the greatest observed egg-laying response from *Delia radicum* at the 1×10^{-4} Molar level. Benzylglucosinolate and prop-*2*-enyl glucosinolate were found to have optimum observed concentrations at 1×10^{-6} and 1×10^{-4} Molar respectively. They were found to stimulate oviposition to a similar degree with benzylglucosinolate eliciting a slightly greater maximum observed response. A comparison of the findings of Roessingh and co-workers (1992) for these 3 glucosinolates with the present study shown in Table 6 (compounds 1 to 3) shows

that the optimum concentrations are similar although the degrees of stimulation do differ. However, the bioassay of Roessingh *et al.* (1992) lacked standardisation in a number of areas most importantly in the number and ages of flies used which could affect the number of eggs laid. Also, only observed maximas of the data were reported by Roessingh *et al.* (1992) whereas this study uses a computer model to "predict" actual optimum concentrations and maximum responses.

Simmonds *et al.*, (1994) studied the neural mechanisms involved in the behaviour of the turnip fly (*Delia floralis* Fallen). Electrophysiological responses were obtained from the long contact sensilla on the labellum as well as type A and D sensilla on the prothoracic and mesothoracic tarsi. As with the cabbage root fly the D_3 and D_4 tarsal sensilla on the prothoracic leg were more sensitive to the glucosinolates than D sensilla on the mesothoracic leg, type A tarsal sensilla or the labellar sensilla. Contact chemoreceptor sensilla were also located on the proboscis of *Delia floralis*. Comparison with the results of Roessingh *et al.* (1992) showed that *D. radicum* was more responsive to glucosinolates than *D. floralis*.

Simmonds and co-workers (1994) found that indol-3-yl methylglucosinolate produced the greatest degree of stimulation of *D. floralis* with an observed optimum concentration of 1×10^{-4} Molar. Prop-2-enylglucosinolate was also highly active with an observed optimum concentration of 1×10^{-5} Molar. Benzylglucosinolate had an optimum observed concentration at 1×10^{-4} Molar although it had a comparatively low observed maximum response.

Indol-3-yl methylglucosinolate is one of the predominate components in the leaves of Brussels sprouts (*B. oleracea* L. var. *gemmifera* DC.), turnips (*B. campestris* L. var. *rapifera* Metzg. Sinsk.), swede (*B. napobrassica* L. Reichenb.) and cabbage (*B. oleracea*

L.) all of which are major host plants for *Delia radicum*. Prop-*2*-enylglucosinolate is also a significant component of the aforementioned crops but to a lesser degree than indol-*3*-yl methylglucosinolate. Trace amounts of benzylglucosinolate have only been detected in cabbage and turnips and various species of mustard (Fenwick *et al.*, 1983; Spinks *et al.* 1984). It would seem that the cabbage root fly has adapted to be more responsive to compounds, such as indol-*3*-yl methylglucosinolate and prop-*2*-enylglucosinolate, which are relatively more abundant in potential host-plants than to compounds only present in trace amounts, such as benzylglucosinolate. This is supported by the work of Roessingh and co-workers (1992) who found that in addition to indol-*3*-yl methylglucosinolate the D_3 and D_4 sensilla were very sensitive to methylglucosinolate, phenethylglucosinolate and pent-*4*-enylglucosinolate which are also major components of brassica crops, especially swede and cabbage (Fenwick *et al.*, 1983; Spinks *et al.*1984). It would also seem that *Delia floralis* has undergone a similar adaptation. Of the eleven glucosinolates tested by Simmonds *et al.* (1994) the turnip root flies were most responsive to indol-*3*-yl methylglucosinolate, but-*3*-enylglucosinolate and pent-*4*-enylglucosinolate, all of which are present in significant amounts in turnips and other brassica crops (Fenwick *et al.*, 1983; Spinks *et al.*1984).

Carbohydrate moieties are present in many secondary metabolites. In compounds such as glucosinolates and cyanogenic glycosides they may act as a protecting group by blocking the rearrangement of the corresponding aglycones to toxic compounds (isothiocyanates and hydrogen cyanide repectively). This would ensure that a reservoir of toxin is safely stored in the plant and only released when the aglycone is produced following predator action. Roessingh *et al.* (1997) showed that the D_3 and D_4 sensilla were sensitive to glucose, fructose and sucrose although the effect of these carbohydrates on oviposition was not studied.

As carbohydrates can readily be modified by protecting some or all of the OH groups it was decided to examine the role of the glucose residue in glucosinolate activity.

In this study modification of the thioglucose residue of prop-*2*-enylglucosinolate by acetylating all of the hydroxyl groups to give potassium *2,3,4,6*-tetra-*O*-acetylprop-*2*-enylglucosinolate (compound 4) caused a decrease in activity from $Y_{MAX} = 2.82 \pm 0.16$ to $Y_{MAX} = 1.17 \pm 0.06$ respectively. The level was however well above the threshold of non-stimulus. This implied that the glucose residue was probably not a key oviposition structural requirement. Thioglucose (compound 5) was bioassayed and found to be inactive. Therefore whole modification of the carbohydrate moeity only leads to a decrease in activity, the sugar alone did not stimulate oviposition.

Using the method of Minchinton *et al.* (1982), the oxime sulphate group was removed using the enzyme aryl sulphatase and resulted in the formation of *S*-(*β*-D-glucopyranosyl)-prop-*2*-enylthiohydroximate (compound 6). This compound was devoid of all activity. Following this interesting observation it was decided to synthesise glucosinolate molecules lacking the oxime sulphate group.

Thus both *S*-(*β*-D-glucopyranosyl)-benzylthiohydroximate and its fully acetylated form, *S*-(*2,3,4,6*-tetra-*O*-acetyl-*β*-D-glucopyranosyl) benzylthiohydroximate were synthesised (compounds 7 and 8). The oxime sulphate group is absent in these compounds and both of them failed to stimulate oviposition. The glucosinolate analogue, *S*-(*3,5*-dimethylphenyl) benzylthiohydroximate (9) which is also unsulphated was synthesised and bioassayed and found to be biologically inactive. The sulphate group seems to be an essential structural feature for oviposition; a region which could be identified as having a crucial role to play possibly as an "on-off switch" for egg laying.

Städler and Shoni (1990) produced evidence that more than one group of compounds stimulates oviposition in *D. radicum*. Roessingh and co-workers (1992), reported that water soluble compounds additional to glucosinolates play a role in the stimulation of oviposition. However the compounds were not identified.

It has been shown that a pair of ventro-medial C sensilla on segment 5 of the tarsus of both the cabbage root fly and turnip fly are sensitive to both glucosinolates and a water soluble, non-glucosinolate, leaf surface extract now known as Cabbage Identification Factor (CIF) but not to glucose, sucrose or fructose (Roessingh *et al.*, 1992b; Baur *et al.*, 1996; Roessingh *et al.*, 1997). In some flies a single neurone in each sensillum is activated by both CIF and glucosinolates and mixtures of both types produce a mononeural spike train whereas in others, spikes of two separate cells were activated. CIF does not evoke responses in the glucosinolate sensitive D type sensilla (Roessingh *et al.*, 1997).

Hopkins *et al.*, (1997) studied the role of surface compounds influencing the oviposition of the turnip fly using bioassays and fractionation of leaf surface extracts. The study compared the oviposition response to the water soluble non-glucosinolate fraction (CIF) of the leaf surface extract with the response to the glucosinolate containing fraction. It was concluded that as with the cabbage root fly, CIF is the major stimulus for turnip fly oviposition.

S-methyl cysteine sulphoxide (10) is known to be present in the leaves of a variety of brassicae including cabbage at significant levels (Morris and Thompson, 1956; Synge and Wood, 1956). Accordingly the group of compounds *S*-methyl cysteine sulphoxide (10), its sulphone (11) and *S*-methyl cysteine (12) were examined.

From the results for *S*-methyl cysteine sulphoxide (10), its sulphone (11) and *S*-methyl cysteine (12) it becomes quite clear that the key structural requirement for

oviposition stimulus is the presence of just an S=O group in a molecule. This group is present in *S*-methyl cysteine sulphoxide and *S*-methyl cysteine sulphone (11) which were bioactive. *S*-methyl cysteine was devoid of biological activity and lacks the S=O group.

In view of the oviposition stimulating ability of *S*-methyl cysteine sulphoxide on surrogate leaves it is possible that it could have the same property *in vivo*. Prop-*2*-enylglucosinolate and *S*-methyl cysteine sulphoxide were tested together on surrogate leaves at varying concentrations (Table 10) to investigate the possibility of synergism. If there was no synergism then the observed stimulation would be equal to the sum of the relative stimulations for each compound at their respective concentrations.

Due to the cubic nature of the oviposition data observed in this study any differences in the concentrations of test solutions applied to surrogate leaves would produce greater differences in the number of eggs laid the nearer the concentrations are to the optimum (C). For this reason the concentrations used to investigate the possibility of synergism were chosen so that they were away from the optimum concentrations for prop-*2*-enylglucosinolate and *S*-methyl cysteine sulphoxide. Any increases above the expected (additive) relative stimulations for the mixtures would then be due to synergism rather than the effects of small experimental errors in the production and application of the test solutions.

When analysed using the appropriate t-test there was no statistical differences between the expected relative stimulations (additive) and observed results at the (P=5%) confidence level (Appendix B). It can be concluded, therefore, that there is no syngergism between *S*-methyl cysteine sulphoxide and prop-*2*-enylglucosinolate.

COMPOUNDS	INITIAL CONCENTRATION (M)	% USED IN TEST MIXTURE	CONCENTRATION IN TEST MIXTURE (M)	EXPECTED Y (ADDITIVE)	ACTUAL Y

PROP-*2*-ENYL GLUCOSINOLATE	1×10^{-5}	90	9×10^{-6}	2.31	2.00
S-METHYL CYSTEINE SULPHOXIDE	1×10^{-4}	10	1×10^{-5}		
PROP-*2*-ENYL GLUCOSINOLATE	1×10^{-5}	50	5×10^{-6}	1.78	1.72
S-METHYL CYSTEINE SULPHOXIDE	1×10^{-4}	50	5×10^{-5}		
PROP-*2*-ENYL GLUCOSINOLATE	1×10^{-5}	10	1×10^{-6}	1.05	0.98
S-METHYL CYSTEINE SULPHOXIDE	1×10^{-4}	90	9×10^{-5}		

TABLE 10 **SYNERGISM RESULTS FOR PROP-*2*-ENYLGLUCOSINOLATE AND *S*-METHYL CYSTEINE SULPHOXIDE**

A series of compounds containing sulphur atoms in various states of oxidation bonded to a variety of other organic residues or functional groups was then examined to test the S=O requirement hypothesis. Table 6, compounds 13 to 24, shows that all of the compounds examined which contained the S=O group possessed significant biological activity despite the wide variety of structures attached to the group.

Hydroxylamine-*O*-sulphonic acid (17) which contains the atom system $\rangle N\text{-}O\text{-}SO_3H$ almost identical to that present in glucosinolates ($=N\text{-}O\text{-}SO_3^- K^+$) was shown to be an effective stimulant. However the most effective compound in terms of Y_{MAX} was the simple compound sulphamic acid (16), in which the sulphur atom of the SO_3 group is bonded directly to an amino-nitrogen atom.

Toluene-*p*-sulphonic acid was tested as both a free sulphonic acid (23) and as the potassium salt (24). The appropriate t-test showed that there was no statistical difference (Appendix B) in the bioassay results for each form at the (P=5%) level showing that it is not necessary to have the ionised SO_3^- group rather than the parent acid. It is assumed from this

that there would also be no significant effect on the activity of those test compounds which could be present as dipolar ions, eg cysteic acid (14), taurine (15) and sulphamic acid (16).

There is also no statistical difference using the appropriate t-test at (P=5%) between toluene-*p*-sulphonic acid (23) and toluene-*p*-sulphonamide (22). Therefore, despite the latter having an amino-nitrogen bonded directly to the sulphur atom, there is no significant increase in activity as was found with hydroxylamine-*O*-sulphonic acid (17) and sulphamic acid (16).

An examination of the biological activity of sodium bisulphite (26) and sodium bisulphate (27) in which the S atom is in its maximum oxidation state showed that both lacked activity suggesting that the S=O group must be covalently and not ionically bonded to the rest of the molecule.

Attempts to sulphate the oxime group of simple ketones to produce compounds more strictly analogous to glucosinolates were unsuccessful, the reaction producing an amide by a spontaneous Beckmann rearrangement of the desired product. This result was not unexpected, since the oxime OH is converted into a good leaving group on sulphation and such compounds were used in early studies on the Beckmann rearrangement.

Roessingh *et al.* (1992) had found a significant correlation between the overall length of the glucosinolate side chain and stimulatory activity.Whether or not this arose from a steric or hydrophobic effect was not discussed.

Octane sulphonic acid (18) and toluene-*p*-sulphonic acid (23) stimulate oviposition to a lesser extent than cysteic acid (14), taurine (15), sulphamic acid (16) and hydroxylamine-*O*-sulphonic acid (17) which seems to show that hydrophobicity of the group adjacent to the SO_3H group is not a dominant factor but the electronegativity of the attached group may be.

There is a close similarity in atomic radii of sulphur and phosphorus atoms (1.09 Å and 1.23 Å respectively), and S=O and P=O bond lengths (1.45 Å and 1.505 Å). For these reasons α-Glucose-*1*-phosphate, disodium salt (32) was bioassayed. Despite the apparent similarities in bond characteristics the phosphated sugar with its P=O failed to exhibit any oviposition simulating properties.

The results show quite clearly the role of a S=O bond attached to an organic residue as an "on/off" switch for oviposition stimulation. However the maximum relative stimulation (Y_{MAX}) ranges from 0.65 to 5.30. It is possible that the bond acts as a "key" fitting into a "lock" on the membrane of chemoreceptors located in sensilla which would then cause a depolarisation of the membrane and the transmission of a signal or neural spike through the nervous system. The presence of sensilla sensitive to the as yet uncharacterised non-glucosinolates in the CIF fraction would explain the stimulatory activity of some of the compounds tested in this study although many are not known to be naturally present in cabbage root fly host-plants. It is possible that the "lock" on the membranes of D_3 and D_4 sensilla require both S=O and a glucose "keys" present in glucosinolates to stimulate a response. However the membrane "lock" for the C_5 sensilla which is sensitive to both non-glucosinolates and glucosinolates but not sugar residues may only require the S=O "key" for stimulation.

The wide range of biological activity between the different test compounds could be due to variations in the configuration of the S=O "key" resulting in differences in the degree of "lock and key" fits at the membrane surface and subsequent neural spike transmission.

A three dimensional computer model of prop-*2*-enylglucosinolate was produced using CS Chem3D and the bond angles compared with X-ray crystallographic data reported by Marsh and Waser (1970) to assess the accuracy of the software. All the bond angles and

lengths matched exactly the values quoted in the literature. Computer models of all the active test compounds were then produced and the bond angles and lengths of the sulphur - oxygen bonds compared. Figures 54, 55 and 56 shows the variation in angle sizes around the sulphur atom for all the test compounds containing SO_3, SO_2 and SO respectively. The sulphur atoms are shown in yellow and the oxygen atoms are in red. It can be seen that there is little variation in the angles within the different group types and between the group types there are certain similarities. The sulphur - oxygen bond lengths in all the test compounds was 1.45 ± 0.01 Å.

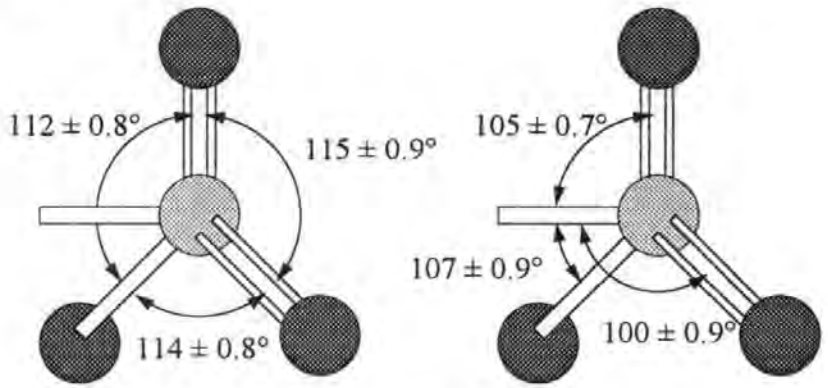


FIGURE 55 VARIATIONS OF BOND ANGLES AROUND THE SULPHUR ATOM IN ALL THE TEST COMPOUNDS CONTAINING THE SO_3 GROUP

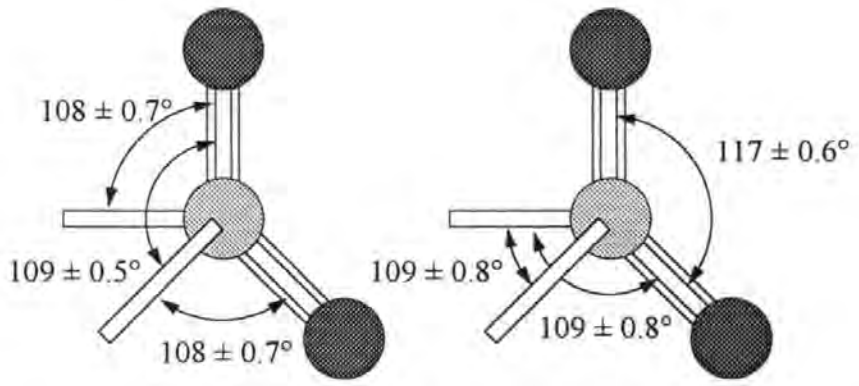


FIGURE 56 VARIATIONS OF BOND ANGLES AROUND THE SULPHUR ATOM IN ALL THE TEST COMPOUNDS CONTAINING THE SO_2 GROUP

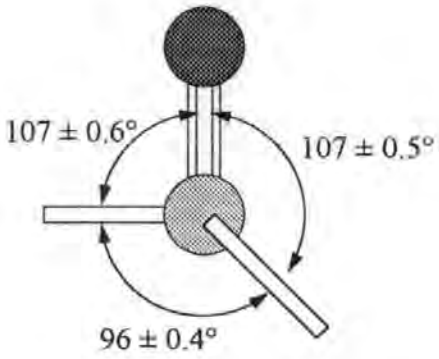


FIGURE 57 **VARIATIONS OF BOND ANGLES AROUND THE SULPHUR ATOM IN ALL THE TEST COMPOUNDS CONTAINING THE SO GROUP**

It can be concluded that whereas an S=O is responsible for a compound to be active the observed variations in activity are due to the nature of the organic residue. This is illustrated clearly in the results obtained for the natural glucosinolates, in each case the molecule differs only in the nature of the R side chain. Indol-3-yl methylglucosinolate has been shown to be the most effective in terms of number of eggs laid relative to a standard (Y_{MAX}) and it was decided to investigate further the role of the indole group.

Ideally it would have been advantageous to synthesise derivatives of indol-3-yl methylglucosinolate but due to the lack of time at this point lengthy syntheses were not possible. Instead it was decided to investigate the properties of some indole compounds containing an S=O group with different distances and geometry relating the two structural elements.

Although indole (33) itself was not biologically active its derivatives were, though to differing degrees. The sodium bisulphite product of indole-3-acetaldehyde (33) produced a higher Y_{MAX} than the sodium bisulphite product of acetone (1.08 and 0.70 respectively). Indoxyl-*3*-sulphate, potassium salt (35) had the highest Y_{MAX} of all the non-glucosinolates tested and proved to have a biological activity greater than that of the natural glucosinolates: prop-*2*-enylglucosinolate and benzylglucosinolate. The N-tosylation of

indole to toluene-*p*-sulphonic acid led to a decrease in maximum relative stimulation from 1.10 for the free acid to 0.66 for the tosylated compound (36).

Principle component analysis is a chemometrics approach thatattempts to identify correlations within a set of variables by employing a number of multi-dimensional statistical and mathematical transformations. It aims to determine linear combinations of the original variables which can be used to summarise the data set without losing much information. Principle component analysis extracts the dominant patterns in the data set looking for outliers and strong groupings. Principle component analysis of the active compounds, their biological activity and degree of stimulation using chemometrics software failed to identify any form of grouping or relationship.

6. CONCLUSIONS

6.1 SUMMARY

The bioassay procedure developed provides a sound and quantifiable method of assessing the efficacy of compounds as oviposition stimulants for the cabbage root fly. The need for a wax layer to be present on the leaf before oviposition occurs indicates that not only must the leaf structure fit certain visual criteria but must also have the correct surface texture. This concurs with results obtained by Roessingh & Städler (1990).

It can be seen that when prop-*2*-enylglucosinolate was tested at the 2.5 x 10^{-5} M level on its own it produced a higher response than when it was used as a standard in with other test compounds, even itself. This suggests that despite being a relatively steady value the number of eggs laid on the standard during bioassay tests is not a true representation of prop-*2*-enylglucosinolate's stimulatory power at the 2.5 x 10^{-5} M level. The results obtained for the standard may be the result of a choice being made by the flies. This highlights the necessity to not only analyse the raw egg count data but to take into consideration the number of eggs laid on both the standards and the blanks.

The results illustrate the role of a S=O bond attatched to an organic residue as an "on/off" switch for oviposition stimulation and that the glucose residue is not a key structural requirement.

Attempts to identify a furthur clear relationship between the test compounds and their activity have been unsuccessful. Three dimensional molecular modelling using CS Chem3D has shown little differences in the configuration of sulphur - oxygen bonds between all the active test compounds. Therefore the wide range of biological activities cannot be attributed to variations in a S=O "key" fitting into a "lock" on the membrane of

the chemoreceptors. Preliminary principle Component analysis of the compounds and their biological activity and degree of stimulation failed to identify any form of grouping or relationship.

The results from experiments to date show that indol-*3*-yl methylglucosinolate, seems to be one of the more important chemical characteristics of the host plant, as is the case also for several other Brassicae related insects such as *Pieris rapae* (Traynier & Truscott, 1990; Renwick *et al.*, 1992), *Pieris napi oleracea* (Huang *et al.*, 1994) and *Delia floralis* (Simmonds *et al.*, 1994). This along with the high degree of stimulation exhibited by the non-glucosinolate, indoxyl-3-sulphate potassium salt, suggests that the indole group has important function to play in stimulating oviposition in the cabbage root fly.

6.2 Further Work

It is clear from the results obtained that the indole side chain enhances biological activity. It would therefore be sensible to investigate further the structure-activity properties of a wide range of indole containing compounds including various derivatives of indol-*3*-yl methylglucosinolate itself. In addition to the indole compounds it could be interesting to synthesise compounds possessing more than one sulphone, sulphoxide and sulphate groups and assess their efficacy as oviposition stimuli.

Studies of electrophysiological responses from the D_3, D_4, A, labellar, C_5 and proboscis sensilla to a range of known active test compounds would provide further insight into the neurological processes connected with host-plant acceptance and oviposition. Research into the biochemical nature of the receptor sites may lead to a better understanding of host-plant acceptance and oviposition at a moleular level.

Work to combine the isothiocyante attractant and glucosinolate stimulant could lead to the development of a commercial system that would lure cabbage root flies away from Brassicae crop and induce them to lay their eggs on a substitute host-plant.

APPENDICES

APPENDIX A

CONCENTRATION OF TEST (M)	N° EGGS LAID ON THE STANDARD (ΣT_1)	N° EGGS LAID ON THE TEST (ΣT_2)	N° EGGS LAID ON THE BLANK (ΣB)
1×10^{-6}	25	9	1
1×10^{-6}	23	11	3
2.5×10^{-6}	22	16	2
2.5×10^{-6}	23	16	3
5×10^{-6}	21	20	0
5×10^{-6}	23	18	1
1×10^{-5}	21	24	1
1×10^{-5}	24	22	3
2.5×10^{-5}		28	1
2.5×10^{-5}		31	0
2.5×10^{-5}		33	0
5×10^{-5}	20	35	1
5×10^{-5}	22	37	3
5×10^{-5}	21	40	2
5×10^{-5}	16	37	3
1×10^{-4}	18	46	2
1×10^{-4}	15	44	1
2.5×10^{-4}	17	48	0
2.5×10^{-4}	16	49	1
2.5×10^{-4}	19	55	2
2.5×10^{-4}	16	52	3
5×10^{-4}	19	47	3
5×10^{-4}	20	48	2
1×10^{-3}	21	43	1
1×10^{-3}	19	44	1
2.5×10^{-3}	17	32	2
2.5×10^{-3}	22	34	3
2.5×10^{-3}	20	37	2
5×10^{-3}	21	29	0
5×10^{-3}	23	18	3
1×10^{-2}	25	14	1
1×10^{-2}	23	18	0

TABLE I RAW BIOASSAY DATA FOR PROP-2-ENYLGLUCOSINOLATE

CONCENTRATION OF TEST (M)	N° EGGS LAID ON THE STANDARD (ΣT_1)	N° EGGS LAID ON THE TEST (ΣT_2)	N° EGGS LAID ON THE BLANK (ΣB)
2.5×10^{-7}	21	21	3
2.5×10^{-7}	23	19	1
1×10^{-6}	20	29	1
1×10^{-6}	22	34	2
1×10^{-6}	19	32	0
2.5×10^{-6}	22	35	1
2.5×10^{-6}	22	43	1
2.5×10^{-6}	20	37	2
5×10^{-6}	21	34	2
5×10^{-6}	21	35	0
1×10^{-5}	23	32	1
1×10^{-5}	21	31	3
2.5×10^{-5}	20	21	3
2.5×10^{-5}	22	24	4
1×10^{-4}	20	16	1
1×10^{-4}	22	17	0
2.5×10^{-4}	19	12	3
2.5×10^{-4}	23	10	1
5×10^{-4}	22	6	2
5×10^{-4}	24	7	2

TABLE II RAW BIOASSAY DATA FOR BENZYLGLUCOSINOLATE

CONCENTRATION OF TEST (M)	N° EGGS LAID ON THE STANDARD (ΣT_1)	N° EGGS LAID ON THE TEST (ΣT_2)	N° EGGS LAID ON THE BLANK (ΣB)
2.5×10^{-7}	21	37	1
2.5×10^{-7}	22	41	3
1×10^{-6}	18	43	0
1×10^{-6}	19	47	1
1×10^{-5}	17	59	3
1×10^{-5}	17	62	4
2.5×10^{-5}	16	70	1
2.5×10^{-5}	17	68	3
5×10^{-5}	15	75	2
5×10^{-5}	14	72	1
1×10^{-4}	15	76	2
1×10^{-4}	14	78	1
2.5×10^{-4}	16	74	2
2.5×10^{-4}	15	75	1
5×10^{-4}	17	70	2
5×10^{-4}	19	73	0
1×10^{-3}	18	65	0
1×10^{-3}	20	68	0
2.5×10^{-3}	20	50	2
2.5×10^{-3}	19	52	1

TABLE III RAW BIOASSAY DATA FOR INDOL-*3*-YLGLUCOSINOLATE

CONCENTRATION OF TEST (M)	Nº EGGS LAID ON THE STANDARD (ΣT_1)	Nº EGGS LAID ON THE TEST (ΣT_2)	Nº EGGS LAID ON THE BLANK (ΣB)
1×10^{-6}	23	19	2
1×10^{-6}	23	17	1
5×10^{-6}	22	21	2
5×10^{-6}	23	22	0
1×10^{-5}	22	24	2
1×10^{-5}	23	23	2
5×10^{-5}	21	25	3
5×10^{-5}	22	26	2
1×10^{-4}	19	24	1
1×10^{-4}	19	25	1
5×10^{-4}	22	20	2
5×10^{-4}	22	21	0
1×10^{-3}	23	16	1
1×10^{-3}	20	15	2

TABLE IV RAW BIOASSAY DATA FOR POTASSIUM *2,3,4,6*-TETRA-*O*-ACETYLPROP-*2*-ENYLGLUCOSINOLATE

CONCENTRATION OF TEST (M)	Nº EGGS LAID ON THE STANDARD (ΣT_1)	Nº EGGS LAID ON THE TEST (ΣT_2)	Nº EGGS LAID ON THE BLANK (ΣB)
1×10^{-7}	20	14	0
1×10^{-7}	19	15	1
1×10^{-6}	22	23	0
1×10^{-6}	21	22	0
1×10^{-5}	19	18	0
1×10^{-5}	21	20	3
2.5×10^{-5}	21	16	0
2.5×10^{-5}	20	18	2
1×10^{-4}	20	15	1
1×10^{-4}	19	14	1
1×10^{-3}	22	11	0
1×10^{-3}	20	10	2

TABLE VRAW BIOASSAY DATA FOR *S*-METHYL CYSTEINE SULPHOXIDE

CONCENTRATION OF TEST (M)	N° EGGS LAID ON THE STANDARD (ΣT_1)	N° EGGS LAID ON THE TEST (ΣT_2)	N° EGGS LAID ON THE BLANK (ΣB)
1×10^{-7}	19	16	0
1×10^{-7}	20	17	1
1×10^{-6}	19	20	2
1×10^{-6}	20	21	0
1×10^{-5}	19	25	0
1×10^{-5}	20	26	1
2.5×10^{-5}	20	27	2
2.5×10^{-5}	21	28	3
1×10^{-4}	22	27	0
1×10^{-4}	20	26	1
1×10^{-3}	21	22	1

TABLE VI RAW BIOASSAY DATA FOR *S*-METHYL CYSTEINE SULPHONE

CONCENTRATION OF TEST (M)	N° EGGS LAID ON THE STANDARD (ΣT_1)	N° EGGS LAID ON THE TEST (ΣT_2)	N° EGGS LAID ON THE BLANK (ΣB)
1×10^{-7}	19	20	1
1×10^{-7}	20	22	0
1×10^{-6}	20	26	3
1×10^{-6}	22	27	2
1×10^{-5}	20	25	0
1×10^{-5}	21	26	1
2.5×10^{-5}	20	24	0
2.5×10^{-5}	22	22	2
1×10^{-4}	21	23	2
1×10^{-4}	20	20	0
1×10^{-3}	21	17	1
1×10^{-3}	20	17	0

TABLE VII RAW BIOASSAY DATA FOR CYSTEINE SULPHINIC ACID

CONCENTRATION OF TEST (M)	Nº EGGS LAID ON THE STANDARD (ΣT_1)	Nº EGGS LAID ON THE TEST (ΣT_2)	Nº EGGS LAID ON THE BLANK (ΣB)
1×10^{-7}	20	21	0
1×10^{-7}	22	22	3
1×10^{-6}	19	29	2
1×10^{-6}	19	30	0
1×10^{-5}	188	27	0
1×10^{-5}	20	29	2
2.5×10^{-5}	21	27	0
2.5×10^{-5}	20	26	2
1×10^{-4}	22	23	3
1×10^{-4}	20	24	1
1×10^{-3}	20	19	1
1×10^{-3}	21	20	0

TABLE VIII RAW BIOASSAY DATA FOR CYSTEIC ACID

CONCENTRATION OF TEST (M)	Nº EGGS LAID ON THE STANDARD (ΣT_1)	Nº EGGS LAID ON THE TEST (ΣT_2)	Nº EGGS LAID ON THE BLANK (ΣB)
1×10^{-7}	21	24	2
1×10^{-7}	20	26	2
1×10^{-6}	19	30	2
1×10^{-6}	20	31	1
1×10^{-5}	20	28	0
1×10^{-5}	19	27	0
2.5×10^{-5}	20	26	1
2.5×10^{-5}	18	24	0
1×10^{-4}	21	23	2
1×10^{-4}	22	23	0
1×10^{-3}	22	20	3
1×10^{-3}	21	19	1

TABLE IX RAW BIOASSAY DATA FOR TAURINE

CONCENTRATION OF TEST (M)	N° EGGS LAID ON THE STANDARD (ΣT_1)	N° EGGS LAID ON THE TEST (ΣT_2)	N° EGGS LAID ON THE BLANK (ΣB)
1×10^{-7}	20	18	3
1×10^{-7}	17	15	2
1×10^{-6}	16	20	0
1×10^{-6}	18	19	2
1×10^{-5}	14	25	0
1×10^{-5}	16	27	1
2.5×10^{-5}	17	31	2
2.5×10^{-5}	15	28	0
1×10^{-4}	14	28	0
1×10^{-4}	16	29	3
1×10^{-3}	20	16	1
1×10^{-3}	19	15	0

TABLE X RAW BIOASSAY DATA FOR SULPHAMIC ACID

CONCENTRATION OF TEST (M)	N° EGGS LAID ON THE STANDARD (ΣT_1)	N° EGGS LAID ON THE TEST (ΣT_2)	N° EGGS LAID ON THE BLANK (ΣB)
1×10^{-7}	22	20	3
1×10^{-7}	20	18	1
1×10^{-6}	22	25	0
1×10^{-6}	21	23	2
1×10^{-5}	18	29	1
1×10^{-5}	20	30	3
2.5×10^{-5}	21	33	2
2.5×10^{-5}	19	31	2
1×10^{-4}	20	30	3
1×10^{-4}	20	31	0
1×10^{-3}	21	18	2
1×10^{-3}	18	19	0

TABLE XI RAW BIOASSAY DATA FOR HYDROXYLAMINE-*O*-SULPHINIC ACID

CONCENTRATION OF TEST (M)	N° EGGS LAID ON THE STANDARD (ΣT_1)	N° EGGS LAID ON THE TEST (ΣT_2)	N° EGGS LAID ON THE BLANK (ΣB)
1×10^{-7}	21	15	0
1×10^{-7}	19	13	2
1×10^{-6}	21	25	1
1×10^{-6}	20	23	2
1×10^{-5}	18	23	1
1×10^{-5}	19	24	1
2.5×10^{-5}	18	22	2
2.5×10^{-5}	22	27	0
1×10^{-4}	20	19	3
1×10^{-4}	20	20	1
1×10^{-3}	19	15	3
1×10^{-3}	21	14	1
1×10^{-2}	18	9	2
1×10^{-2}	17	11	0

TABLE XII RAW BIOASSAY DATA FOR OCTANE SULPHONIC ACID

CONCENTRATION OF TEST (M)	N° EGGS LAID ON THE STANDARD (ΣT_1)	N° EGGS LAID ON THE TEST (ΣT_2)	N° EGGS LAID ON THE BLANK (ΣB)
1×10^{-7}	18	5	2
1×10^{-7}	16	4	1
1×10^{-6}	17	5	0
1×10^{-6}	20	7	2
1×10^{-5}	17	9	0
1×10^{-5}	16	8	1
2.5×10^{-5}	19	11	0
2.5×10^{-5}	17	10	2
1×10^{-4}	18	13	1
1×10^{-4}	17	12	2
1×10^{-3}	16	11	0
1×10^{-3}	17	12	3

TABLE XIII RAW BIOASSAY DATA FOR ACETONE SODIUM BISULPHITE

CONCENTRATION OF TEST (M)	N° EGGS LAID ON THE STANDARD (ΣT_1)	N° EGGS LAID ON THE TEST (ΣT_2)	N° EGGS LAID ON THE BLANK (ΣB)
1×10^{-7}	21	13	2
1×10^{-7}	23	12	0
1×10^{-6}	19	14	3
1×10^{-6}	17	13	2
1×10^{-5}	20	19	0
1×10^{-5}	18	21	2
2.5×10^{-5}	19	25	0
2.5×10^{-5}	21	28	1
1×10^{-4}	22	29	3
1×10^{-4}	17	25	0
1×10^{-3}	20	19	0
1×10^{-3}	18	17	2

TABLE XIV RAW BIOASSAY DATA FOR *1,3,2,λ-6*-BENZODIOXATHIOLE-*2,2*,-DIONE

CONCENTRATION OF TEST (M)	N° EGGS LAID ON THE STANDARD (ΣT_1)	N° EGGS LAID ON THE TEST (ΣT_2)	N° EGGS LAID ON THE BLANK (ΣB)
1×10^{-7}	18	15	0
1×10^{-7}	17	14	1
1×10^{-6}	19	19	2
1×10^{-6}	22	21	0
1×10^{-5}	18	17	1
1×10^{-5}	20	17	1
2.5×10^{-5}	19	16	1
2.5×10^{-5}	18	15	2
1×10^{-4}	21	15	3
1×10^{-4}	22	14	0
1×10^{-3}	19	10	1
1×10^{-3}	18	8	0

TABLE XV RAW BIOASSAY DATA FOR *5*-NITRO-*2,3*-DIHYDRO-*1,2λ-6*-BENZOXATHIOLE-*2,2*-DIONE

CONCENTRATION OF TEST (M)	N° EGGS LAID ON THE STANDARD (ΣT_1)	N° EGGS LAID ON THE TEST (ΣT_2)	N° EGGS LAID ON THE BLANK (ΣB)
1×10^{-7}	20	16	1
1×10^{-7}	19	15	0
1×10^{-6}	17	21	0
1×10^{-6}	18	22	2
1×10^{-5}	19	21	0
1×10^{-5}	18	19	1
2.5×10^{-5}	19	17	1
2.5×10^{-5}	20	19	2
1×10^{-4}	20	14	2
1×10^{-4}	19	16	0
1×10^{-3}	20	12	2
1×10^{-3}	19	13	3

TABLE XVI RAW BIOASSAY DATA FOR TOLUENE-*P*-SULPHONAMIDE

CONCENTRATION OF TEST (M)	N° EGGS LAID ON THE STANDARD (ΣT_1)	N° EGGS LAID ON THE TEST (ΣT_2)	N° EGGS LAID ON THE BLANK (ΣB)
1×10^{-7}	18	10	2
1×10^{-7}	19	9	0
1×10^{-6}	16	11	1
1×10^{-6}	14	10	2
1×10^{-5}	17	15	3
1×10^{-5}	16	15	1
2.5×10^{-5}	16	17	1
2.5×10^{-5}	15	16	2
1×10^{-4}	14	15	2
1×10^{-4}	16	17	0
1×10^{-3}	14	12	2
1×10^{-3}	17	14	1

TABLE XVII RAW BIOASSAY DATA FOR TOLUENE-*P*-SULPHONIC ACID

CONCENTRATION OF TEST (M)	N° EGGS LAID ON THE STANDARD (ΣT_1)	N° EGGS LAID ON THE TEST (ΣT_2)	N° EGGS LAID ON THE BLANK (ΣB)
1×10^{-7}	19	9	0
1×10^{-7}	20	11	0
1×10^{-6}	18	14	3
1×10^{-6}	20	13	1
1×10^{-5}	20	17	0
1×10^{-5}	19	16	1
2.5×10^{-5}	22	19	1
2.5×10^{-5}	19	17	2
1×10^{-4}	20	20	0
1×10^{-4}	19	19	3
1×10^{-3}	19	17	1
1×10^{-3}	20	18	0

TABLE XVIII RAW BIOASSAY DATA FOR POTASSIUM TOLUENE-*P*-SULPHONATE

CONCENTRATION OF TEST (M)	N° EGGS LAID ON THE STANDARD (ΣT_1)	N° EGGS LAID ON THE TEST (ΣT_2)	N° EGGS LAID ON THE BLANK (ΣB)
1×10^{-7}	18	2	1
1×10^{-7}	12	1	0
1×10^{-6}	13	5	1
1×10^{-6}	16	7	2
1×10^{-5}	17	5	0
1×10^{-5}	15	5	0
2.5×10^{-5}	16	7	0
2.5×10^{-5}	14	6	1
1×10^{-4}	12	6	0
1×10^{-4}	13	7	3
1×10^{-3}	14	11	0
1×10^{-3}	16	10	2
1×10^{-2}	18	9	1
1×10^{-2}	17	5	0

TABLE XIX RAW BIOASSAY DATA FOR GRAMINE TOLUENE-*P*-SULPHONATE

CONCENTRATION OF TEST (M)	N° EGGS LAID ON THE STANDARD (ΣT_1)	N° EGGS LAID ON THE TEST (ΣT_2)	N° EGGS LAID ON THE BLANK (ΣB)
1×10^{-7}	19	11	0
1×10^{-7}	21	12	2
1×10^{-6}	18	19	1
1×10^{-6}	19	18	3
1×10^{-5}	22	23	2
1×10^{-5}	19	22	2
2.5×10^{-5}	21	21	0
2.5×10^{-5}	23	21	2
1×10^{-4}	19	17	1
1×10^{-4}	21	18	1
1×10^{-3}	18	12	2
1×10^{-3}	19	11	0
1×10^{-2}	21	8	0
1×10^{-2}	23	9	1

TABLE XX RAW BIOASSAY DATA FOR INDOLE-*3*-ACETALDEHYDE SODIUM BISULPHITE

CONCENTRATION OF TEST (M)	N° EGGS LAID ON THE STANDARD (ΣT_1)	N° EGGS LAID ON THE TEST (ΣT_2)	N° EGGS LAID ON THE BLANK (ΣB)
1×10^{-7}	17	24	0
1×10^{-7}	18	25	1
1×10^{-6}	16	32	0
1×10^{-6}	17	29	3
1×10^{-5}	13	35	2
1×10^{-5}	15	36	3
2.5×10^{-5}	16	41	3
2.5×10^{-5}	15	42	2
1×10^{-4}	16	50	0
1×10^{-4}	15	49	1
1×10^{-3}	14	43	2
1×10^{-3}	13	39	1
1×10^{-2}	18	26	0
1×10^{-2}	14	24	0

TABLE XXI RAW BIOASSAY DATA FOR INDOXYL-*3*-SULPHATE, POTASSIUM SALT

CONCENTRATION OF TEST (M)	N° EGGS LAID ON THE STANDARD (ΣT_1)	N° EGGS LAID ON THE TEST (ΣT_2)	N° EGGS LAID ON THE BLANK (ΣB)
1×10^{-7}	17	9	2
1×10^{-7}	14	8	3
1×10^{-6}	18	13	0
1×10^{-6}	16	11	1
1×10^{-5}	13	8	2
1×10^{-5}	14	10	3
2.5×10^{-5}	14	8	2
2.5×10^{-5}	13	8	3
1×10^{-4}	15	5	0
1×10^{-4}	12	6	1
1×10^{-3}	16	4	0
1×10^{-3}	14	3	0
1×10^{-2}	15	3	2
1×10^{-2}	15	2	0

TABLE XXII RAW BIOASSAY DATA FOR *1*-(TOLUENE-*4*-SULPHONYL)-INDOLE

APPENDIX B

EXAMPLE 1 STATISTICAL ANALYSIS OF REPEAT BIOASSAYS

Statistical analysis of the number of eggs laid on the standards in two repeat bioassays for acetone sodium bisulphite at1 x 10^{-7} M concentration.

Bioassay (a)

4	3	0
2	8	0
2	0	6

Bioassay (b)

7	1	0
0	4	1
2	1	5

T_{1a}	T_{2a}	B_a
4	3	0
8	0	2
0	2	0

T_{1b}	T_{2b}	B_b
7	1	0
4	1	0
5	2	1

A two sample F-test for variances

	T_{1a}	T_{1b}
Mean	*4*	*5.333*
Variance	*16*	*2.333*
Observations	*3*	*3*
df	*2*	*2*
F	*6.857*	
F Critical one-tail	*19.000*	

F < F Critical, therefore there is no significant difference between the variances

t-Test : Two sample assuming equal variances (P=5%)

	T_{1a}	T_{1b}
Mean	*4*	*5.333*
Variance	*16*	*2.333*
Observations	*3*	*3*
Pooled Variance	*9.167*	
Hypothesized Mean Difference	*0*	
df	*4*	
t Stat	*-0.539*	
t Critical one-tail	*8.61*	
t Critical two-tail	*10.31*	

t Stat < t Critical, therefore there is no significant difference between T_{1a} and T_{1b}. It is assumed that there is also no significant differences between the two repeat bioassays

EXAMPLE 2 AN ANALYSIS OF VARIANCE TO DETERMINE ANY BIAS WITHIN THE BIOASSAY ARENA

ANOVA of bioassay results for acetone sodium bisulphite at 1 x 10^{-7}M concentration.

Bioassay (a)		
4	3	0
2	8	0
2	0	6

T_{1a}	T_{2a}	B_a
4	3	0
8	0	2
0	2	0

Anova: Two factor without replication

	Count	Sum	Average	Variance
Row 1	3	8	2.666	14.333
Row 2	3	5	1.666	4.333
Row 3	3	8	2.666	4.333
Column 1	3	9	3	
Column 2	3	6	2	
Column 3	3	6	2	

ANOVA

	SS	df	MS	F	P-value	F Crit
Rows	2	2	1	0.090	0.914	10.649
Columns	2	2	1	0.090	0.914	10.649
Error	44	4	11			
Total	48	8				

F < F Critical for both the rows and columns, therefore there is no significant differences between the numbers of eggs laid in different areas of the bioassay arena., i.e. no bias.

EXAMPLE 3 STATISTICAL ANALYSIS TO DETERMINE SIGNIFICANT POSITIVE OVIPOSITION STIMULATION

Stasistical analysis of the number of eggs laid on the tests (T_{2a}) and the blanks (B) for acetone sodium bisulphite at 1 x 10^{-7} M concentration

Bioassay (a)		
4	3	0
2	8	0
2	0	6

T_{1a}	T_{2a}	B_a
4	3	0
8	0	2
0	2	0

A two sample F-test for variances

	T_{2a}	B_a
Mean	*1.667*	*5.333*
Variance	*2.333*	*2.333*
Observations	*3*	*3*
df	*2*	*2*
F	*1.75*	
F Critical one-tail	*39.000*	

F < F Critical, therefore there is no significant difference between the variances

t-Test : Two sample assuming equal variances (P=5%)

	T_{2a}	B_a
Mean	*1.667*	*5.333*
Variance	*2.333*	*2.333*
Observations	*3*	*3*
Pooled Variance	*1.833*	
Hypothesized Mean Difference	*0*	
df	*4*	
t Stat	*9.045*	
t Critical one-tail	*2.776*	
t Critical two-tail	*3.495*	

t Stat > t Critical, therefore there is a significant difference between T_{2a} and B_a indicating a positive oviposition stimulation by acetone sodium bisulphite at the 1 x 10^{-7} M concentration level.

EXAMPLE 4 STATISTICAL ANALYSIS OF CORRELATION COEFFICIENTS

Determination of the level of significance for the correlation coefficient calculated for *S*-Methyl cysteine sulphone at (P=5%)

R^2 Correlation Coefficient	*0.958*
Observations	*6*
df	*4*
r	*0.958*
r Critical two tail	*0.811*

r > r Critical, therefore there is a significant correlation between Log_{10} Concentration and Relative Stimulation for *S*-Methyl cysteine sulphone

EXAMPLE 5 STATISTICAL COMPARISON OF DATA COLLECTED FOR TOLUENE-*P*-SULPHONIC ACID AND POTASSIUM TOLUENE-*P*-SULPHONATE

TOLUENE-*P*-SULPHONIC ACID (A)

CONCENTRATION OF TEST (M)	RELATIVE STIMULATION (Y)
1×10^{-7}	0.5
1×10^{-7}	0.473
1×10^{-6}	0.666
1×10^{-6}	0.666
1×10^{-5}	0.857
1×10^{-5}	0.933
2.5×10^{-5}	1.066
2.5×10^{-5}	1.076
1×10^{-4}	1.083
1×10^{-4}	1.062
1×10^{-3}	0.833
1×10^{-3}	0.812

POTASSIUM TOLUENE-*P*-SULPONATE (B)

CONCENTRATION OF TEST (M)	RELATIVE STIMULATION (Y)
1×10^{-7}	0.473
1×10^{-7}	0.550
1×10^{-6}	0.733
1×10^{-6}	0.631
1×10^{-5}	0.850
1×10^{-5}	0.833
2.5×10^{-5}	0.857
2.5×10^{-5}	0.882
1×10^{-4}	1
1×10^{-4}	1
1×10^{-3}	0.888
1×10^{-3}	0.9

A two sample F-test for variances

	A	*B*
Mean	*0.836*	*0.800*
Variance	*0.049*	*0.08*
Observations	*12*	*12*
df	*11*	*11*
F	*1.709*	
F Critical one-tail	*2.817*	

F < F Critical, therefore there is no significant difference between the variances

t-Test : Two sample assuming equal variances (P=5%)

	A	*B*
Mean	*0.836*	*0.800*
Variance	*0.049*	*0.08*
Observations	*12*	*12*
Pooled Variance	*0.038*	
Hypothesized Mean Difference	*0*	
df	*22*	
t Stat	*0.449*	
t Critical one-tail	*1.717*	
t Critical two-tail	*2.074*	

t Stat < t Critical, therefore there is no significant difference between Toluene-*p*-sulphonic acid and its potassium salt

EXAMPLE 6 STATISTICAL COMPARISON OF DATA COLLECTED FOR TOLUENE-*P*-SULPHONIC ACID AND TOLUENE-*P*-SULPHONAMIDE

TOLUENE-*P*-SULPHONIC ACID (A)

CONCENTRATION OF TEST (M)	RELATIVE STIMULATION (Y)
1×10^{-7}	
1×10^{-7}	
1×10^{-6}	
1×10^{-6}	
1×10^{-5}	
1×10^{-5}	
2.5×10^{-5}	
2.5×10^{-5}	
1×10^{-4}	
1×10^{-4}	
1×10^{-3}	
1×10^{-3}	

TOLUENE-*P*-SULPONAMIDE (B)

CONCENTRATION OF TEST (M)	RELATIVE STIMULATION (Y)
1×10^{-7}	
1×10^{-7}	
1×10^{-6}	
1×10^{-6}	
1×10^{-5}	
1×10^{-5}	
2.5×10^{-5}	
2.5×10^{-5}	
1×10^{-4}	
1×10^{-4}	
1×10^{-3}	
1×10^{-3}	

A two sample F-test for variances

	A	*B*
Mean	*0.836*	*0.895*
Variance	*0.049*	*0.053*
Observations	*12*	*12*
df	*11*	*11*
F	*1.84*	
F Critical one-tail	*2.817*	

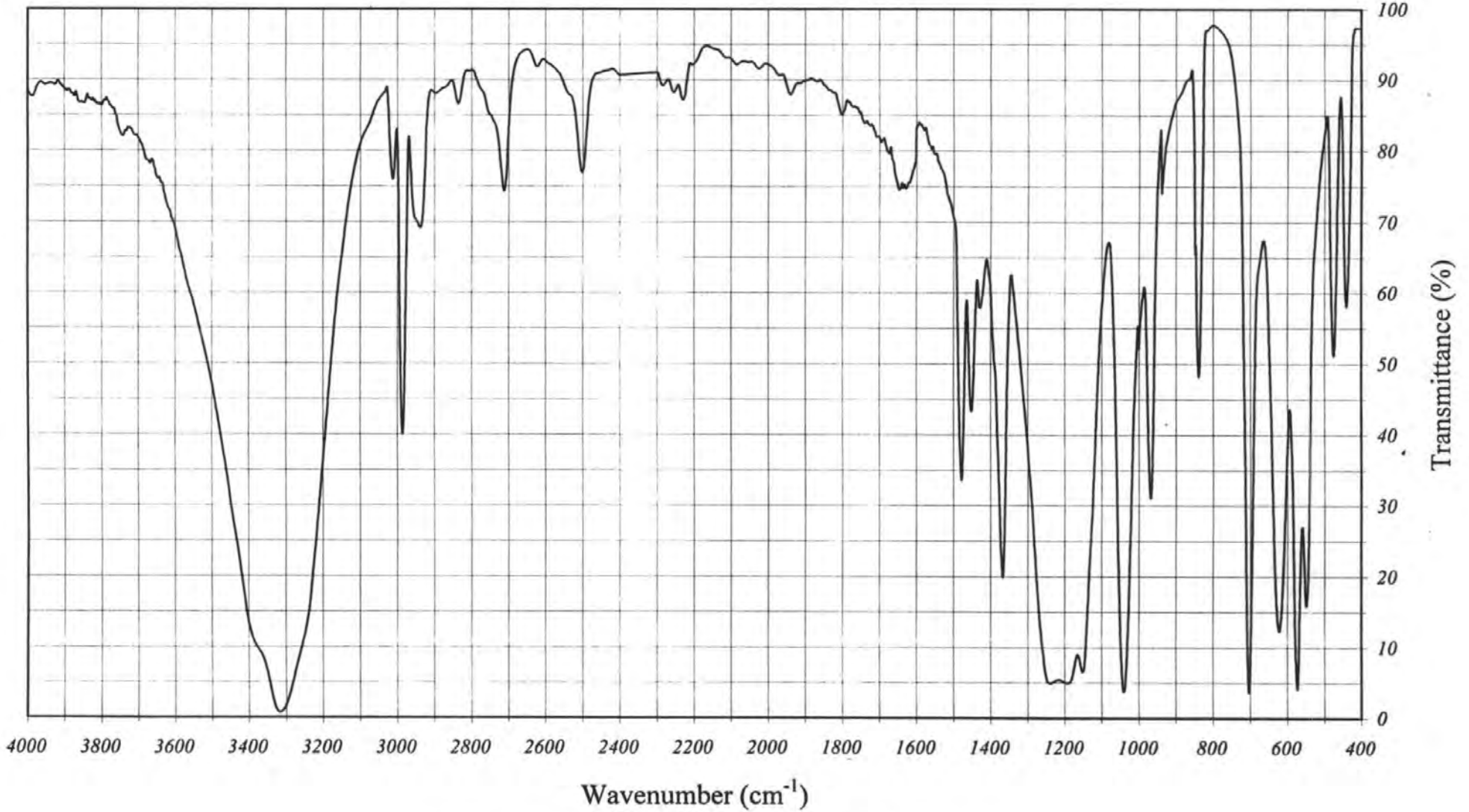
F < F Critical, therefore there is no significant difference between the variances

t-Test : Two sample assuming equal variances (P=5%)

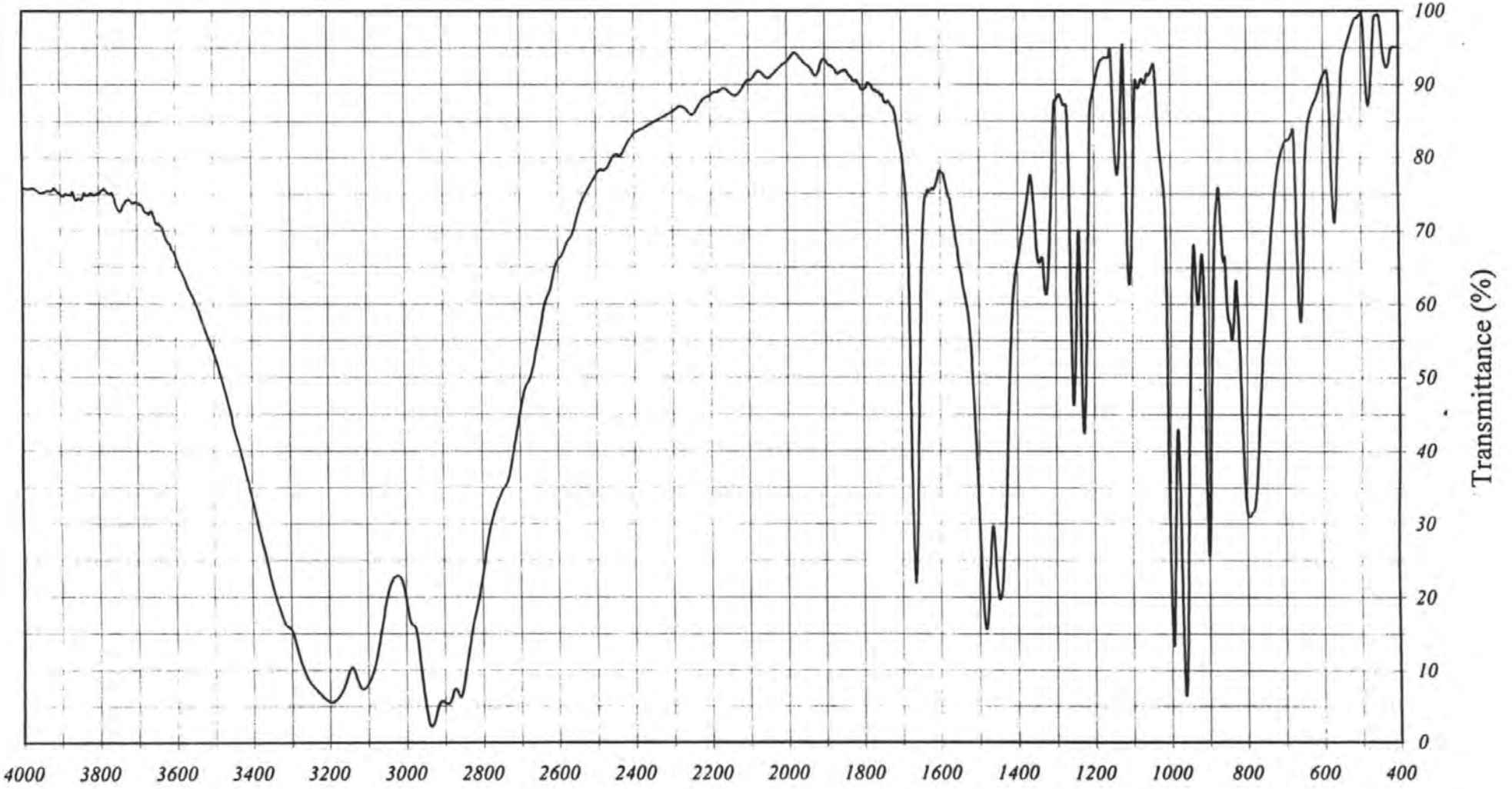
	A	*B*
Mean	*0.836*	*0.895*
Variance	*0.049*	*0.53*
Observations	*12*	*12*
Pooled Variance	*0.050*	
Hypothesized Mean Difference	*0*	
df	*22*	
t Stat	*-0.646*	
t Critical one-tail	*1.717*	
t Critical two-tail	*2.074*	

t Stat < t Critical, therefore there is no significant difference between Toluene-*p*-sulphonic acid and Toluene-*p*-sulphonamide

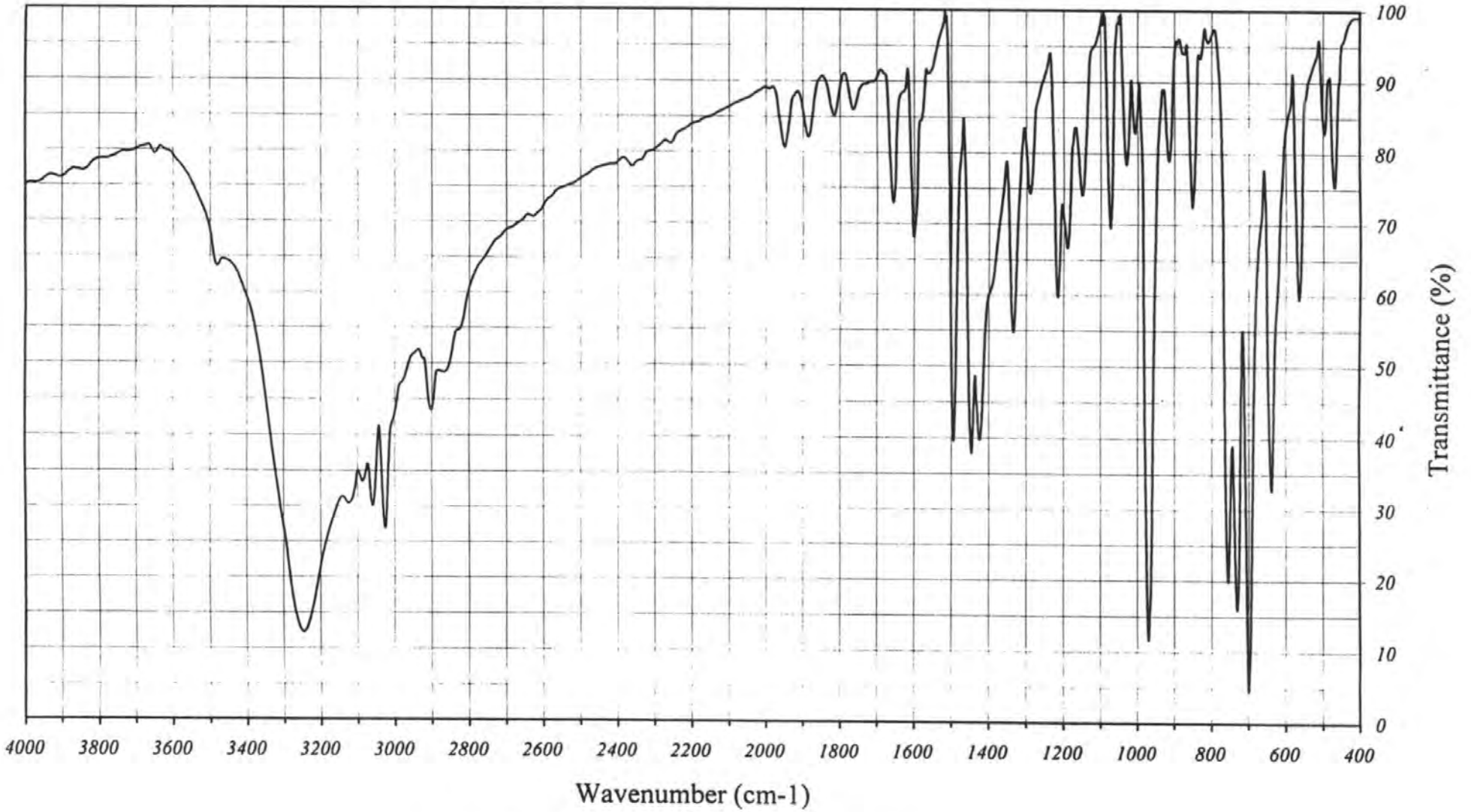
APPENDIX C



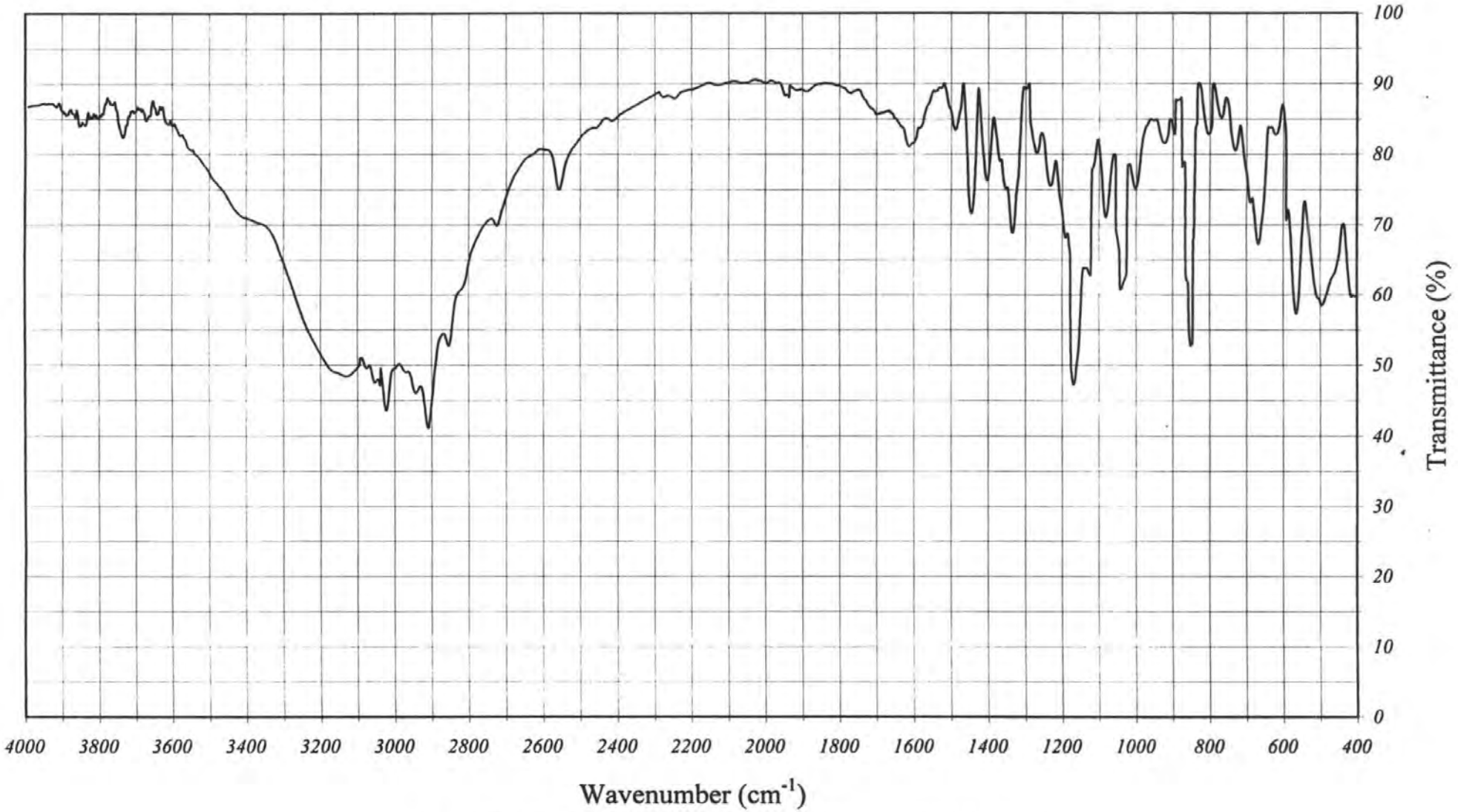
INFRA-RED SPECTRUM FOR ACETONE SODIUM BISULPHITE



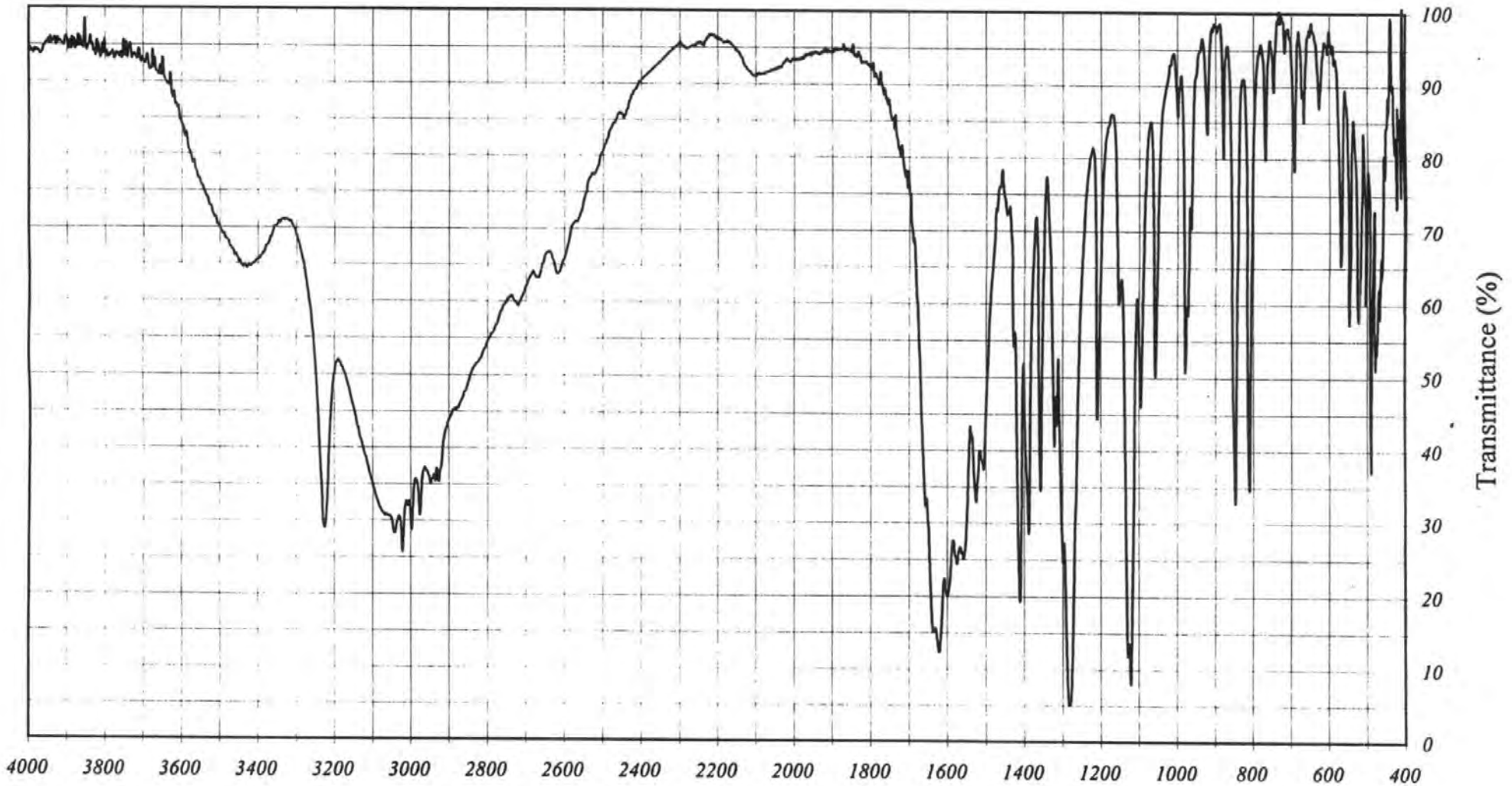
INFRA-RED SPECTRUM FOR CYCLOHEXANONE OXIME



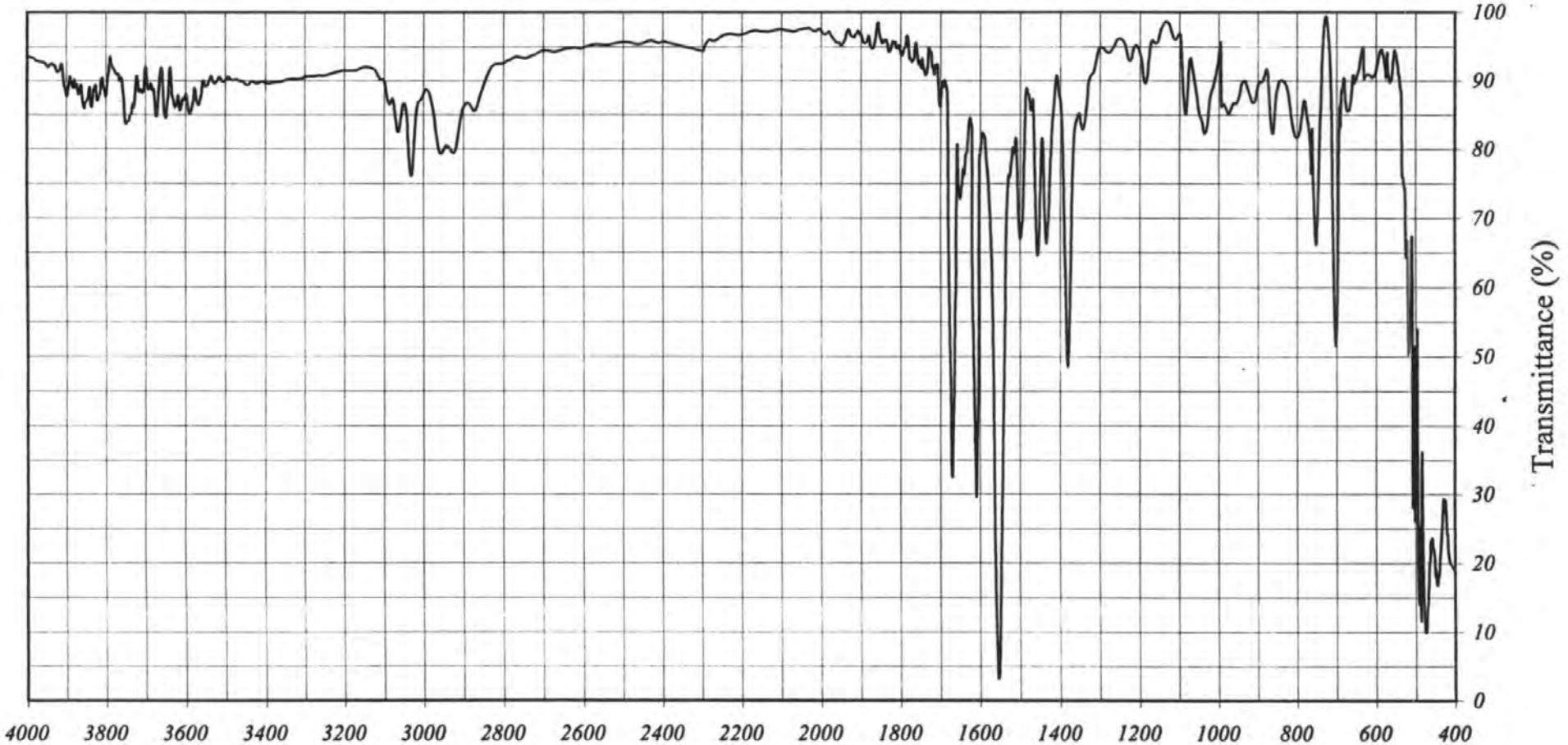
INFR-RED SPECTRUM FOR *1,3*-DIPHENYLACETONE OXIME



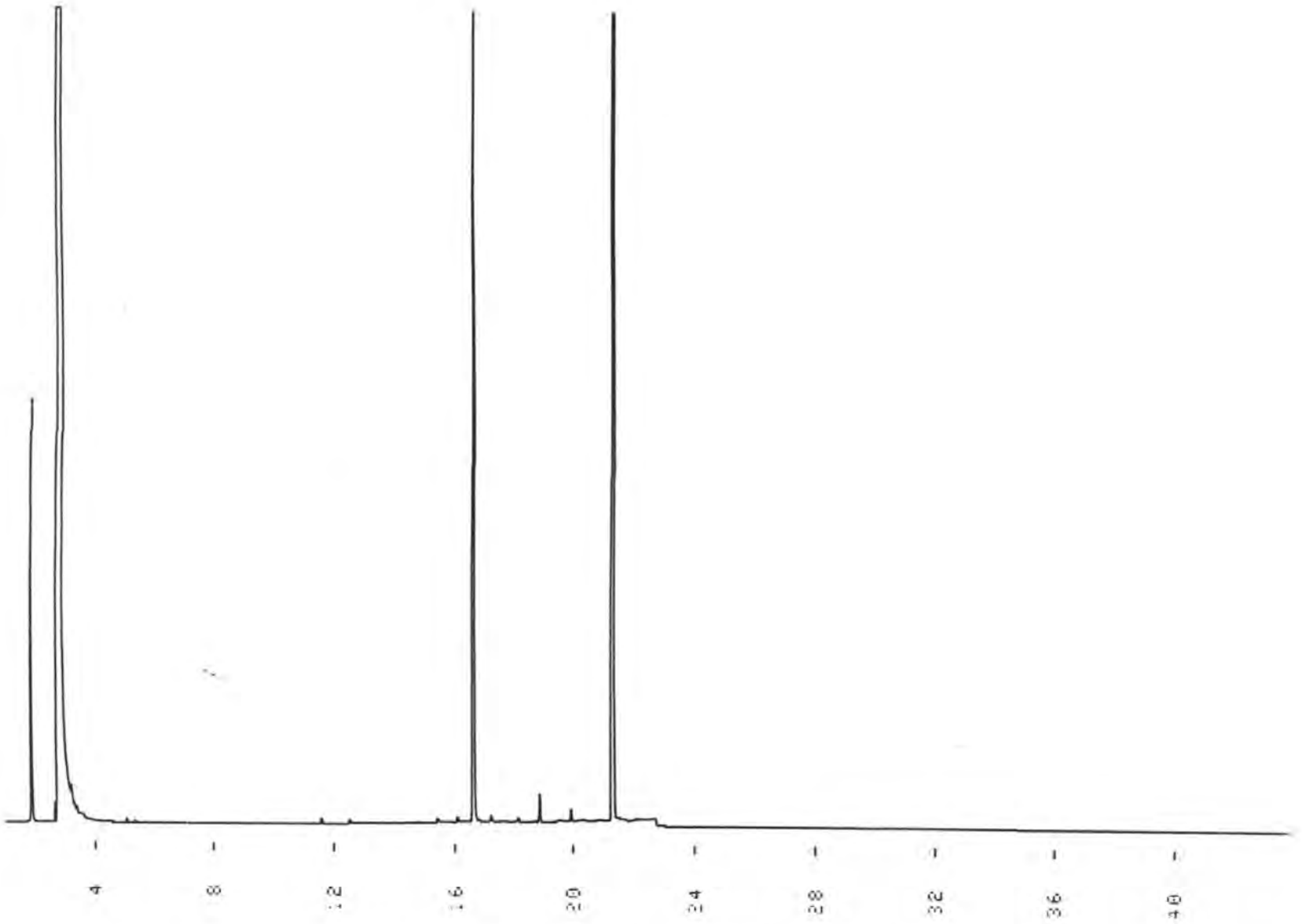
INFRA-RED SPECTRUM FOR *1*-(TOLUENE-*4*-SULPHONYL)-INDOLE



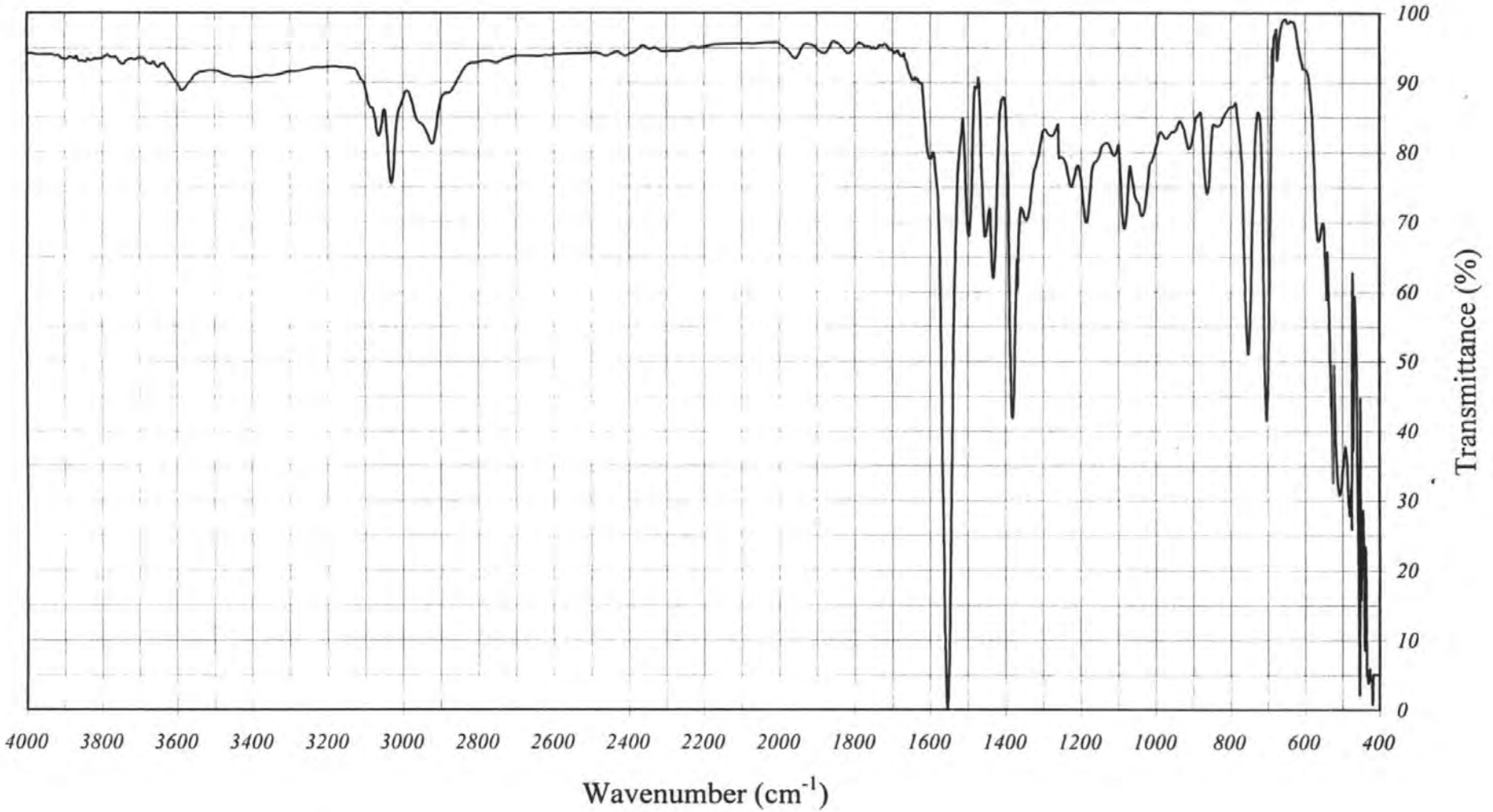
INFRA-RED SPECTRUM FOR *S*-METHYL CYSTEINE SULPHONE



GAS CHROMATOGRAM FOR *2*-NITROETHYL BENZENE (CRUDE)

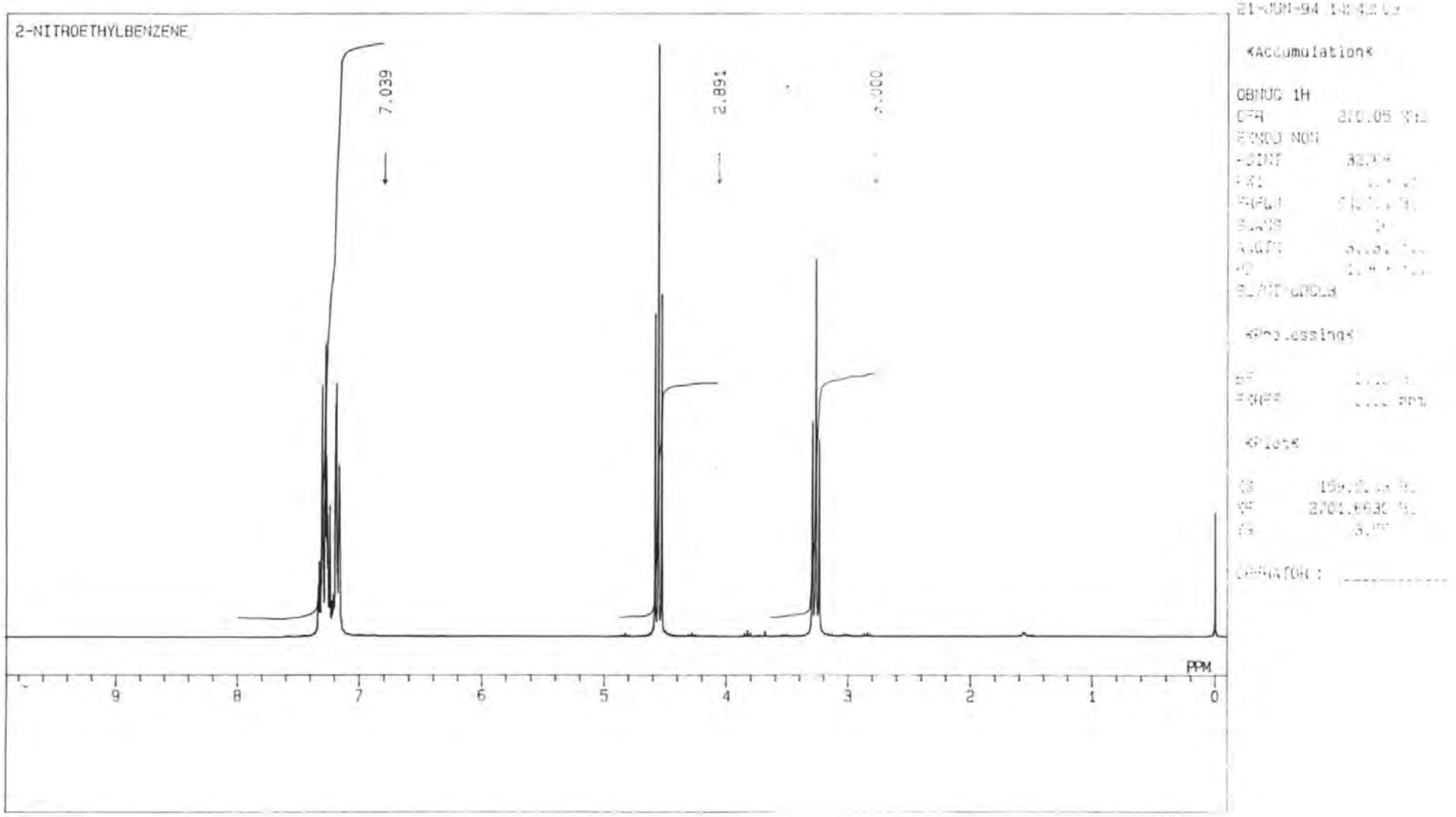


GAS CHROMATOGRAM FOR *2*-NITROETHYL BENZENE (CRUDE)

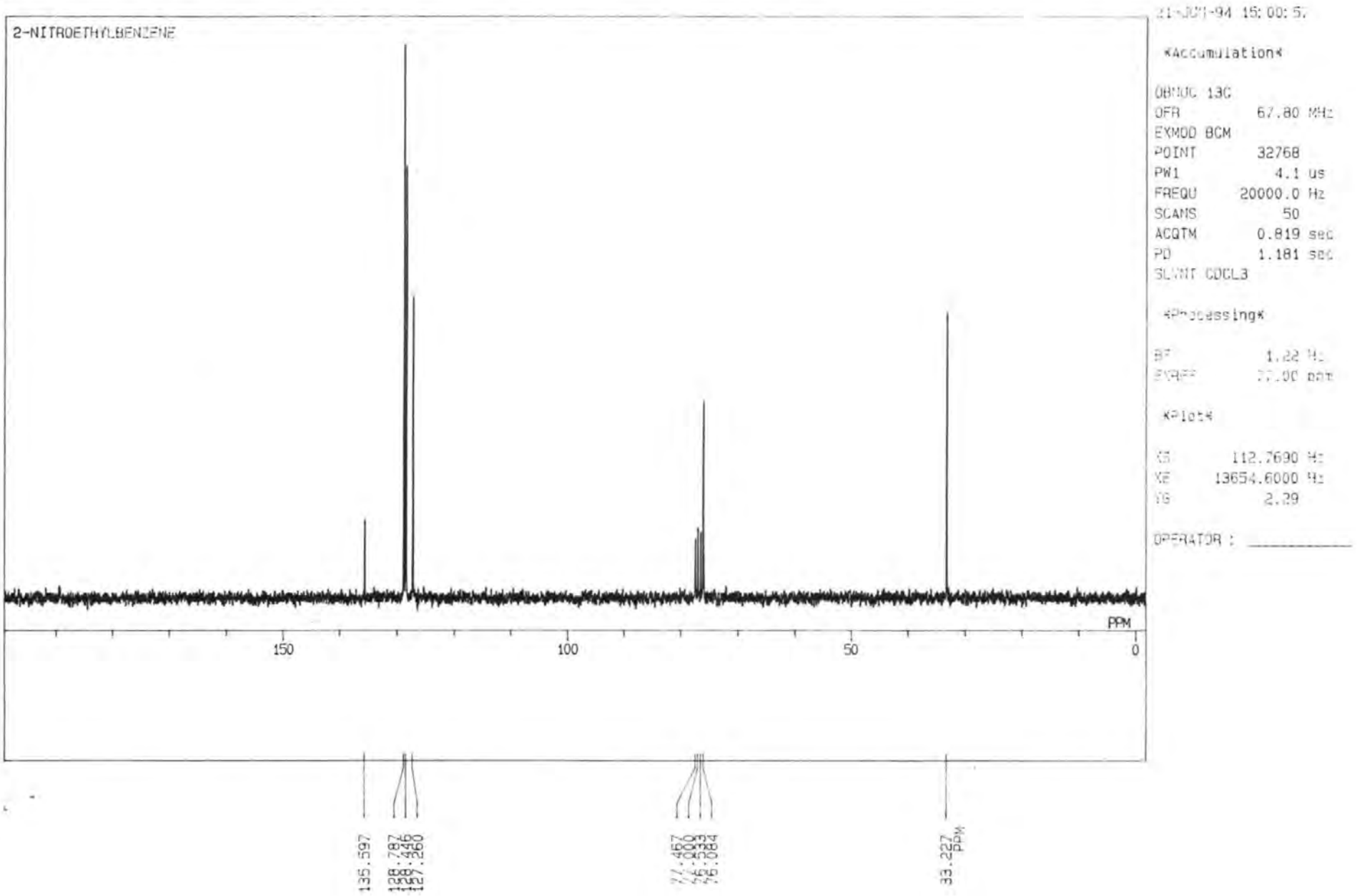


INFRA-RED SPECTRUM FOR *2*-NITROETHYL BENZENE (PURE)

GAS CHROMATOGRAM FOR *2*-NITROETHYL BENZENE (PURE)



^{1}H NMR FOR *2*-NITROETHYL BENZENE (PURE)



^{13}C NMR FOR *2*-NITROETHYL BENZENE (PURE)

Butterworth Laboratories Ltd

ANALYTICAL and CONSULTING CHEMISTS

54-56 WALDEGRAVE ROAD, TEDDINGTON, MIDDLESEX TW11 8LG, U.K.

Telephone: 081-977 0750 Fax: 081-943 2624

NAMAS TESTING No. 0215

ANALYSIS IN COMPLIANCE WITH G.L.P.

APPLICATION FORM FOR MICROANALYSIS Ref. No. BL7/ 0219 (94)

(One sample per Application Form)

Applicants Name D J. BRAVEN

Address: DEPT. OF ENVIRONMENTAL SCIENCES

UNIV. OF PLYMOUTH

DEVON PL 4 8AA

Tel. No. 0752 233000 Extn.

Fax No. 0752 233035

Order No. A/094070/93

Sample No. NC 2

Type and/or Structure

$C_6H_5CH_2CH_2NO_2$

Elements present C, H, N, O

M.Pt/B.Pt. 84°/0.5 Torr

Moisture/Air/Oxygen Sensitive [X]

Drying Required [No]

Dry Box Handling [NC]

Toxic/Carcinogenic [ ] Prop. explosive

Any other hazard [ ]

Any Other Comments.

ANALYSIS REQUIRED

Element	Single	Dupl.	Expected	% Found
C.	✓		63.57	63.37
H.	✓		5.96	6.08
N.	✓		9.27	9.24
F.				
Cl.				
Br.				
I.				
S.				
P.				
Metals				
Others				

Comments by Analyst

Urgent Service [ ] Tel: [ ] Fax Service [ ]

Authorised Signature

DATE SENT 5/07/1994

DATE RECEIVED 7 JUL 1994

DATE REPORTED 11 July 1994

For Company Use only

Analysis in accordance with M.S.1

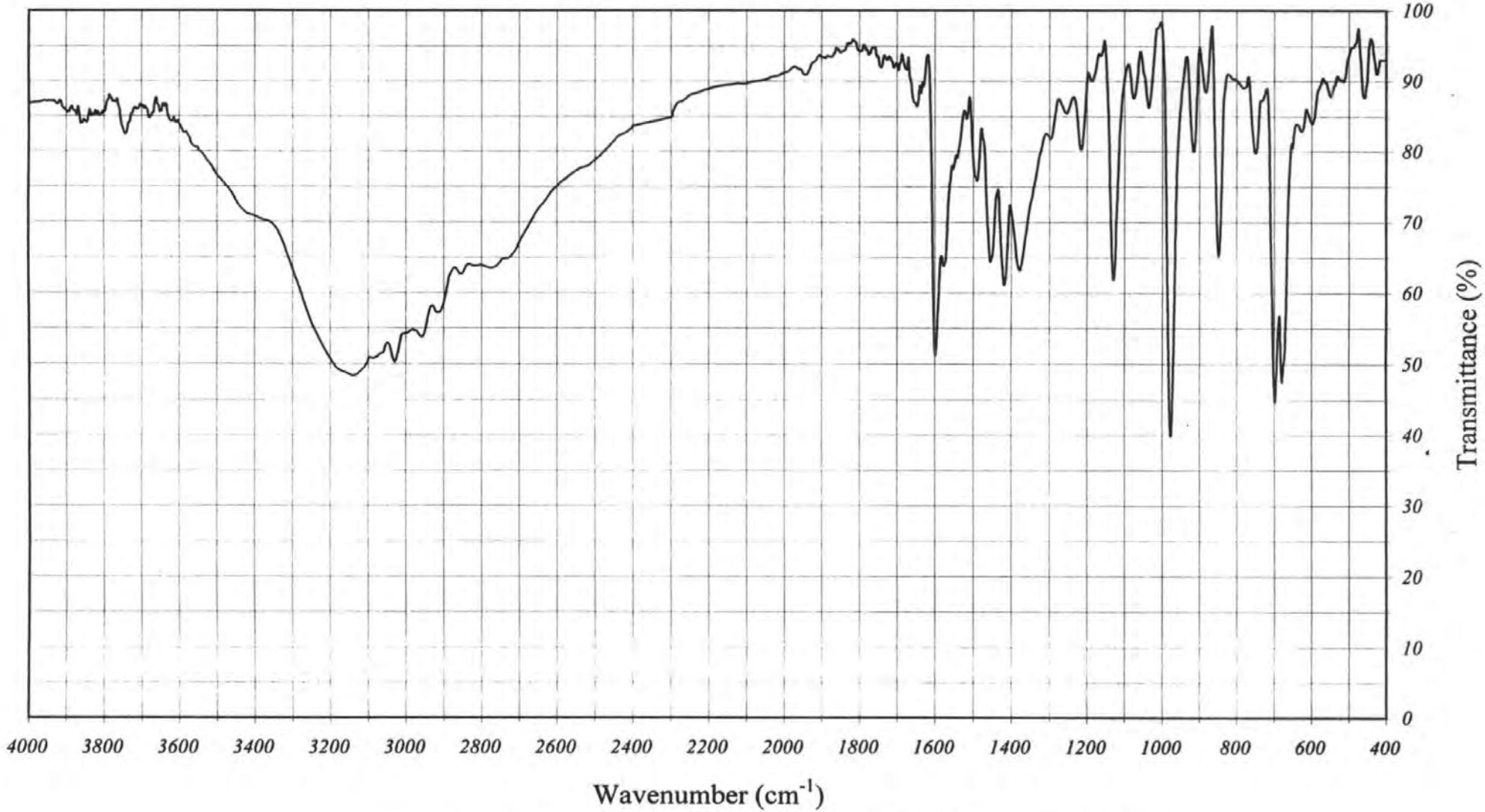
I confirm that this analysis is in compliance with G.L.P. and accept responsibility for the results.

Study Director

The Q.A. Unit audits all analytical results and undertakes periodic monitoring of studies.

Q.A. Department

Approved David Butterworth Managing Director



INFRA-RED SPECTRUM FOR *S*-(*3,5*-DIMETHYLPHENYL) BENZYLTHIOHYDROXIMATE

Butterworth Laboratories Ltd
Analytical & Consulting Chemists

1974 to 1994

54-56 Waldegrave Road,
Teddington, Middlesex
TW11 8LG UK

Telephone: 081-977 0750
Fax: 081-943 2624

GLP
Compliant Laboratory

NAMAS TESTING No 0215

APPLICATION FORM FOR MICROANALYSIS
(One sample per Application Form)

BL11/ 0786 (94)

If GLP Study required please tick box ☐

Butterworth Ref. .....

Applicants Name Dr. J. BRAVEN

Address DEPT. OF ENVIRONMENTAL SCIENCES
UNIVERSITY OF PLYMOUTH
DEVON PL 4 8AA

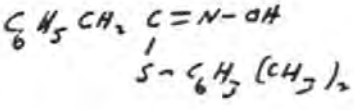
Tel. No. 0752 233010 Extn. .....

Fax No. 0752 233035

Order No. A/09830/94

Sample No. NC 20

Type and/or Structure



ANALYSIS REQUIRED

Element	Minimum sample weight mg.	Single/ Duplicate S/D	% m/m Expected	% m/m Found	
C.	1.5	S	70.85	70.38	70.28
H.		S	6.27	5.98	6.18
N.		S	5.17	5.26	5.47
F.	5				
Cl.	5				
Br.	5				
I.	5				
S.	5				
P.	5				
Metals	10				
Others					
Asn	10				

Elements present C, H, N, O, S

M Pt./B Pt. 110°

Moisture/Air/Oxygen Sensitive ☐ Drying Required ☐

Analyser used: PE2400 ☑ LEEMAN 440 ☐

Comments on Analysis

Safety Information

Toxic ☐ Carcinogenic / Teratogenic ☐

Radioactive ☐ Explosive ☐ Flammable ☐ Pyrophoric ☐ Unknown ☑

Any other information/comments

Analyst .....

For Company Use only

Analysis in accordance with M.S.1.

I confirm that this analysis is in compliance with G.L.P. and accept responsibility for the results.

..... Study Director

The Q.A. Unit audits all analytical results and undertakes periodic monitoring of studies.

..... Q.A. Department

Approved ..... Managing and Technical Director

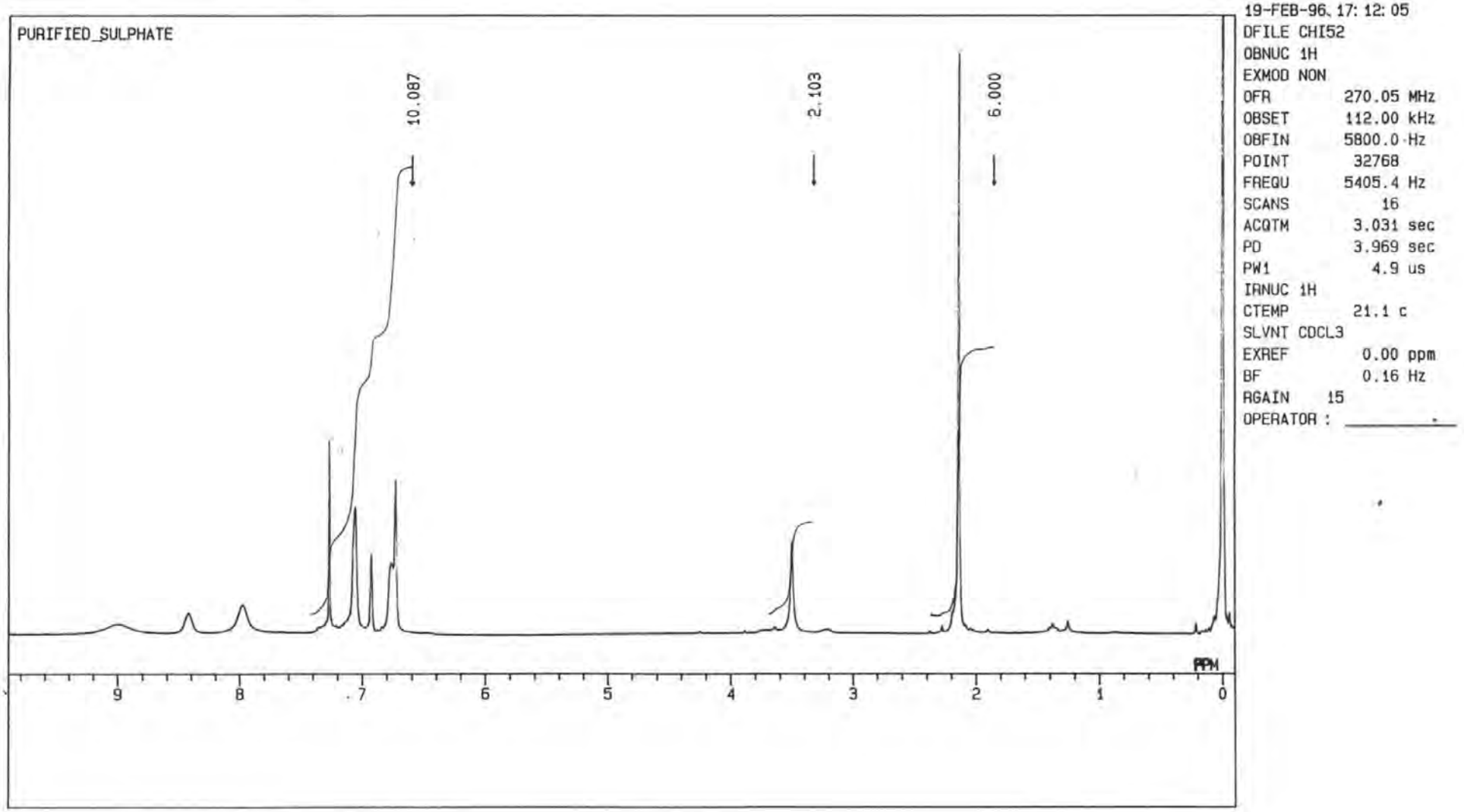
Urgent Service ☐ (Subject to Surcharge) Tel: ☐ Fax ☐

Authorising Signature .....

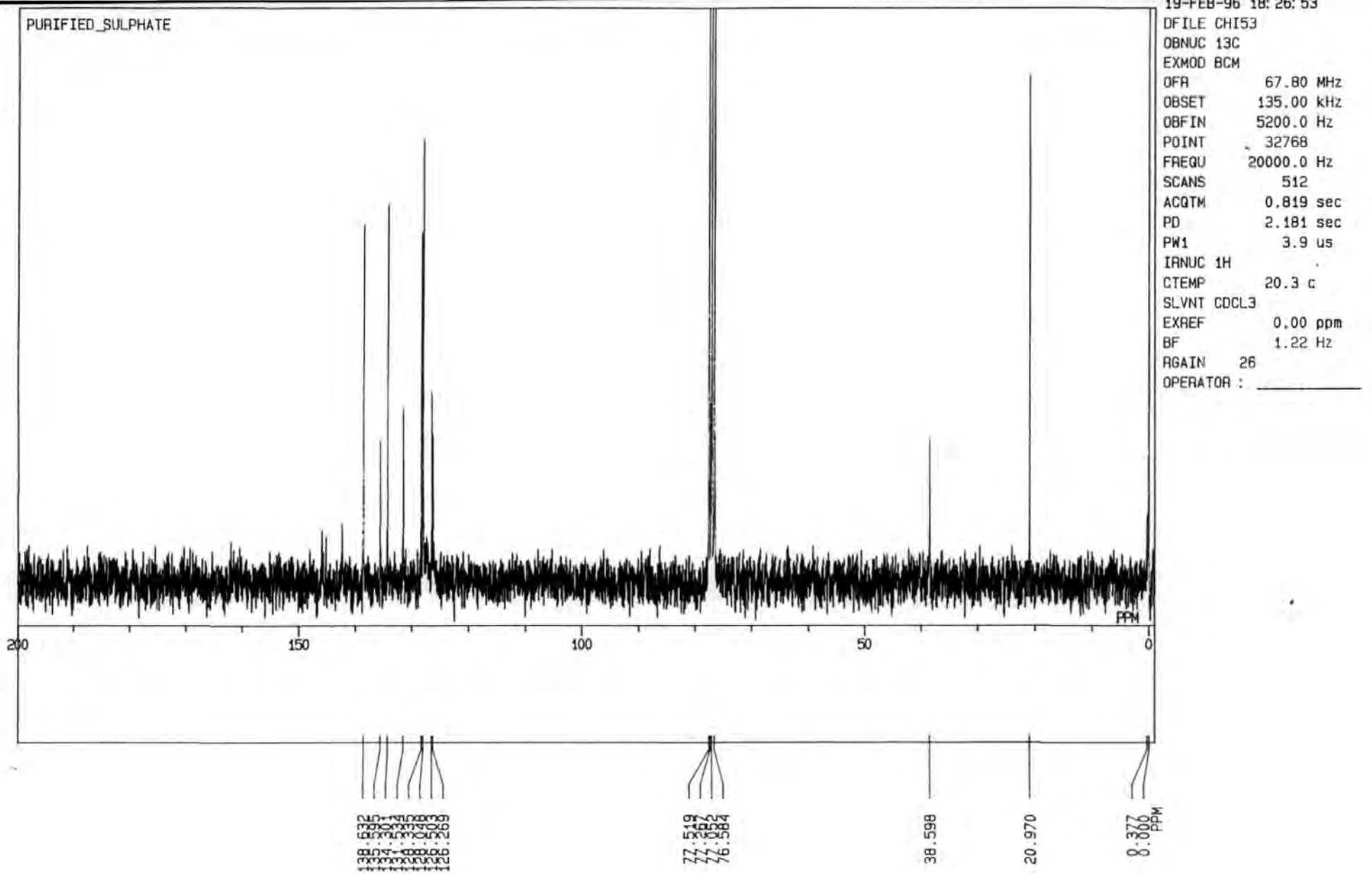
DATE SENT 18.11.94

DATE RECEIVED 21 NOV 1994

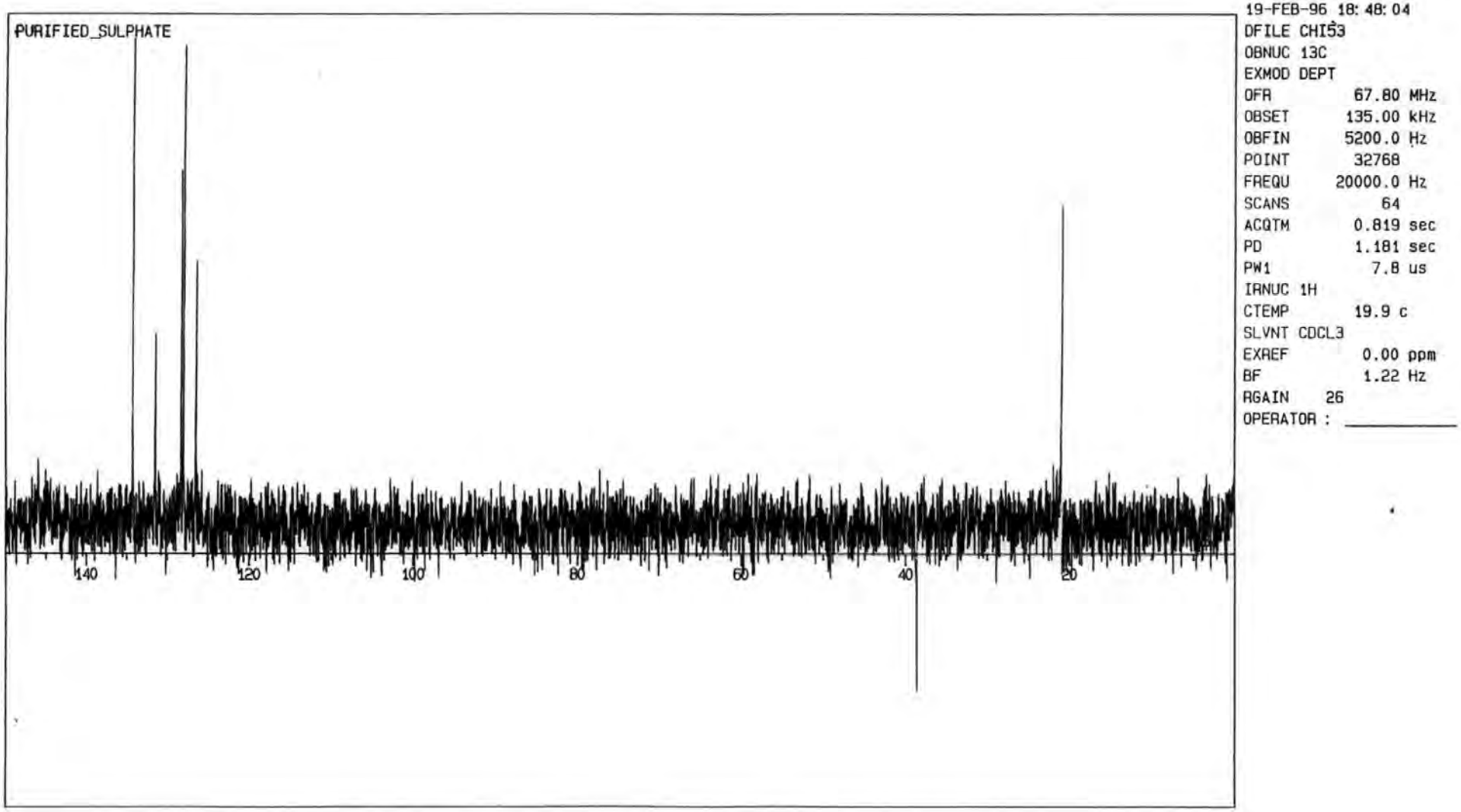
DATE REPORTED 23rd November 1994



^{1}H NMR OF THE PRODUCT ISOLATED FROM THE ATTEMPTED SULPHATION OF *S*-(*3,5*-DIMETHYLPHENYL) BENZYLTHIOHYDROXIMATE



^{13}C NMR OF THE PRODUCT ISOLATED FROM THE ATTEMPTED SULPHATION OF *S*-(*3,5*-DIMETHYLPHENYL) BENZYLTHIOHYDROXIMATE



DEPT NMR OF THE PRODUCT ISOLATED FROM THE ATTEMPTED SULPHATION OF *S*-(*3,5*-DIMETHYLPHENYL) BENZYLTHIOHYDROXIMATE

Butterworth Laboratories Ltd
Analytical & Consulting Chemists

1974 to 1995

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TW11 8LG UK

Telephone: 0181-977 0750
Fax: 0181-943 2624

GLP
Compliant Laboratory

NAMAS TESTING No 0215

APPLICATION FORM FOR MICROANALYSIS
(One sample per Application Form)

If GLP Study required please tick box ☐

Butterworth Ref: BL .....

Applicants Name D J. BRAVEN

Address DEPT OF ENVIRONMENTAL SCIENCES
UNIV. OF PLYMOUTH
DEVON PL4 8AA

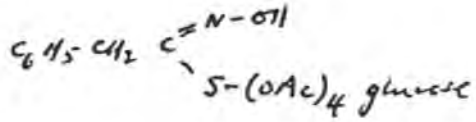
Tel. No. 01752 233010 Extn. .....

Fax No. 01752 233035

Order No. A/118238/95

Sample No. NC 44

Type and/or Structure



Elements present C, H, N, O, S

M Pt./B.Pt. 165°

Moisture/Air/Oxygen Sensitive ☐ Drying Required ☐

Safety Information

Toxic ☐ Carcinogenic / Teratogenic ☐

Radioactive ☐ Explosive Flammable Pyrophoric ☐ Unknown ☑

Any other information/comments

.....

.....

.....

Urgent Service ☐ (Subject to Surcharge) Tel: ☐ Fax ☐

Authorising Signature .....

DATE SENT 13/12/1995

DATE RECEIVED .....

DATE REPORTED .....

ANALYSIS REQUIRED

Element	Minimum sample weight mg.	Single/ Duplicate S/D	% m/m Expected	% m/m Found
C.	1.5	✓	53.12	
H.		✓	5.47	
N.		✓	2.82	
F.	5			
Cl.	5			
Br.	5			
I.	5			
S.	5			
P.	5			
Metals	100			
(Trace)	1g			
Others				
Ash	10			

Analyser used: PE2400 ☐ LEEMAN 440 ☐

Comments on Analysis

Analyst .....

For Company Use only

Analysis in accordance with M.S.1.

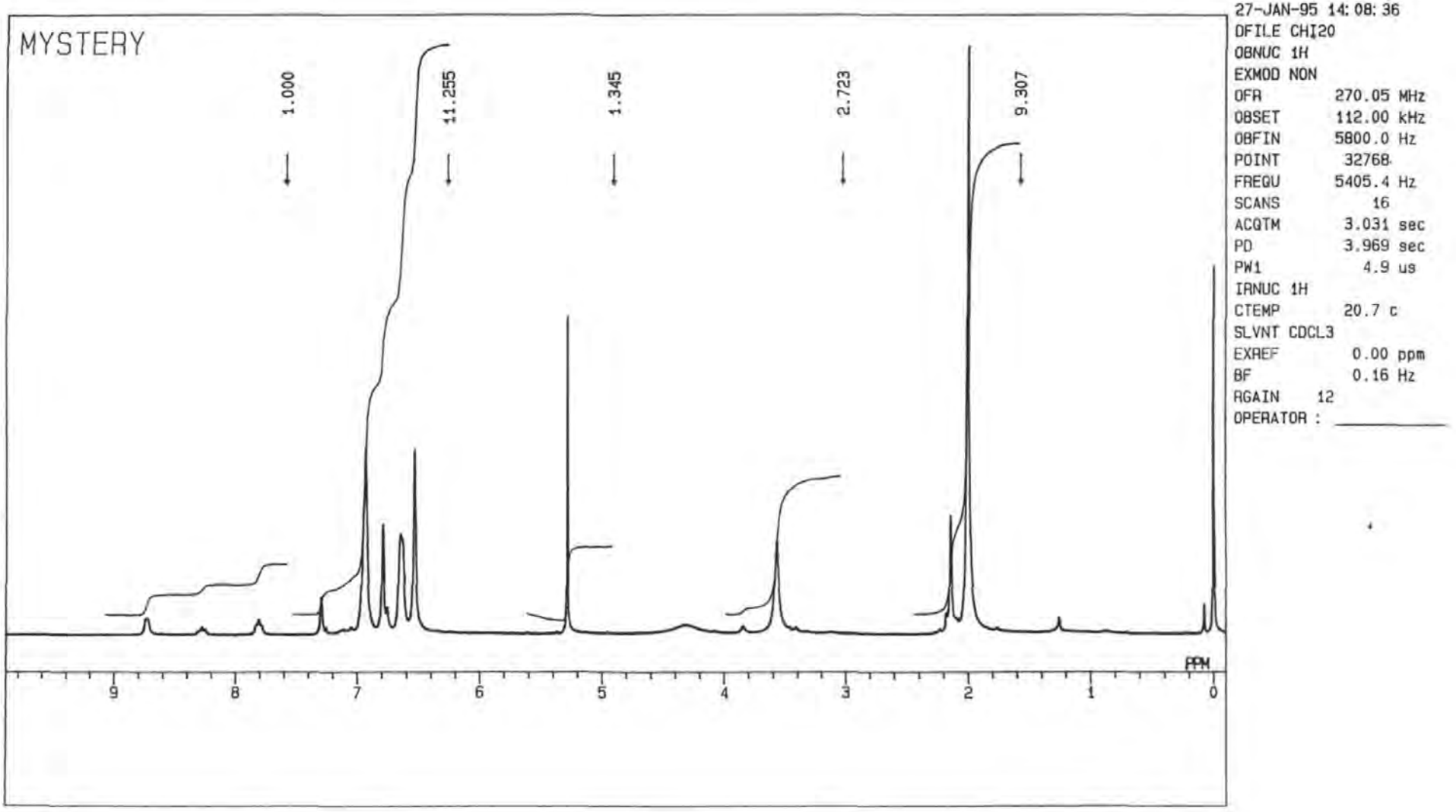
I confirm that this analysis is in compliance with G.L.P. and accept responsibility for the results.

..... Study Director

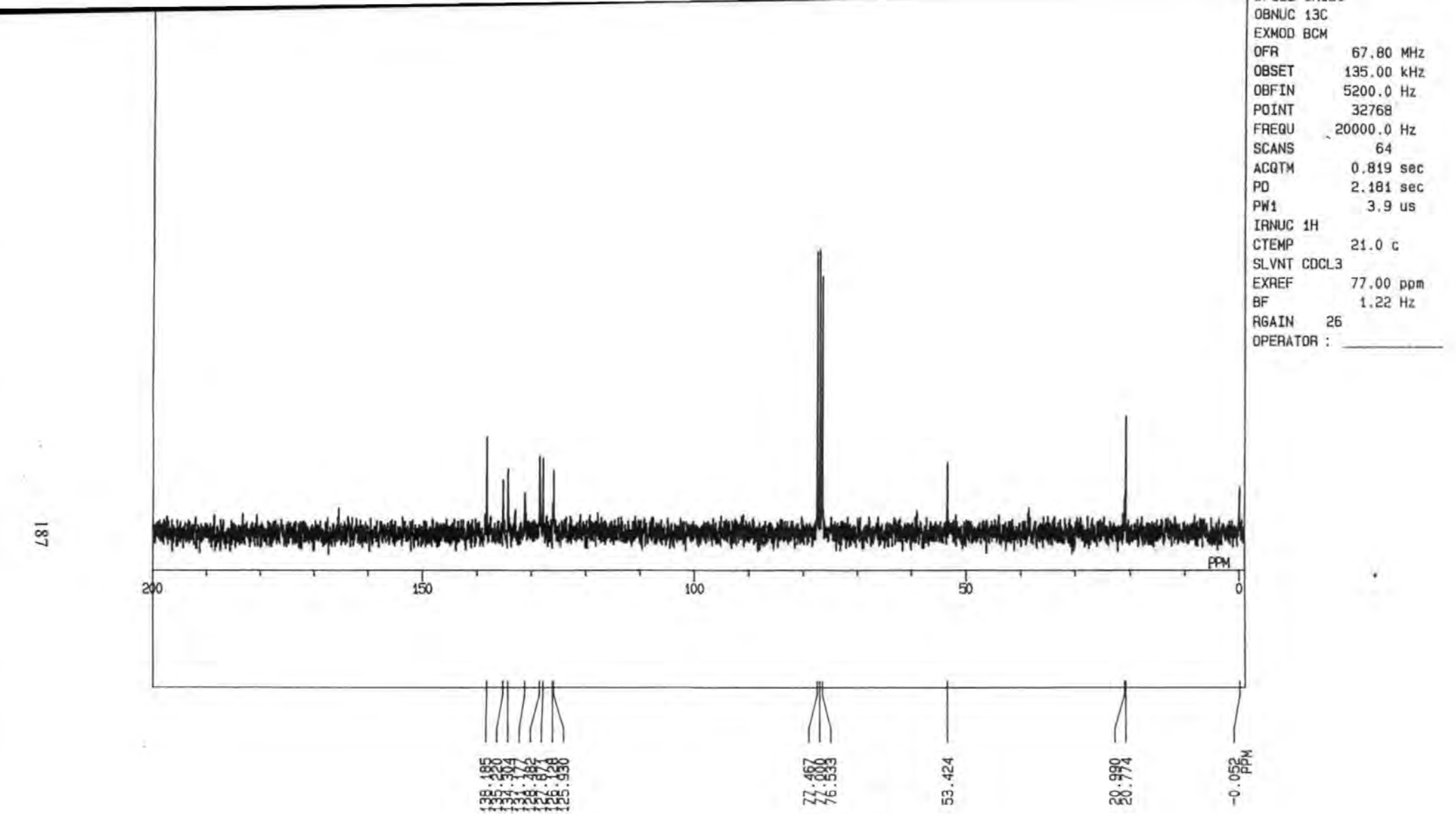
The Q.A. Unit audits all analytical results and undertakes periodic monitoring of studies.

..... Q.A. Department

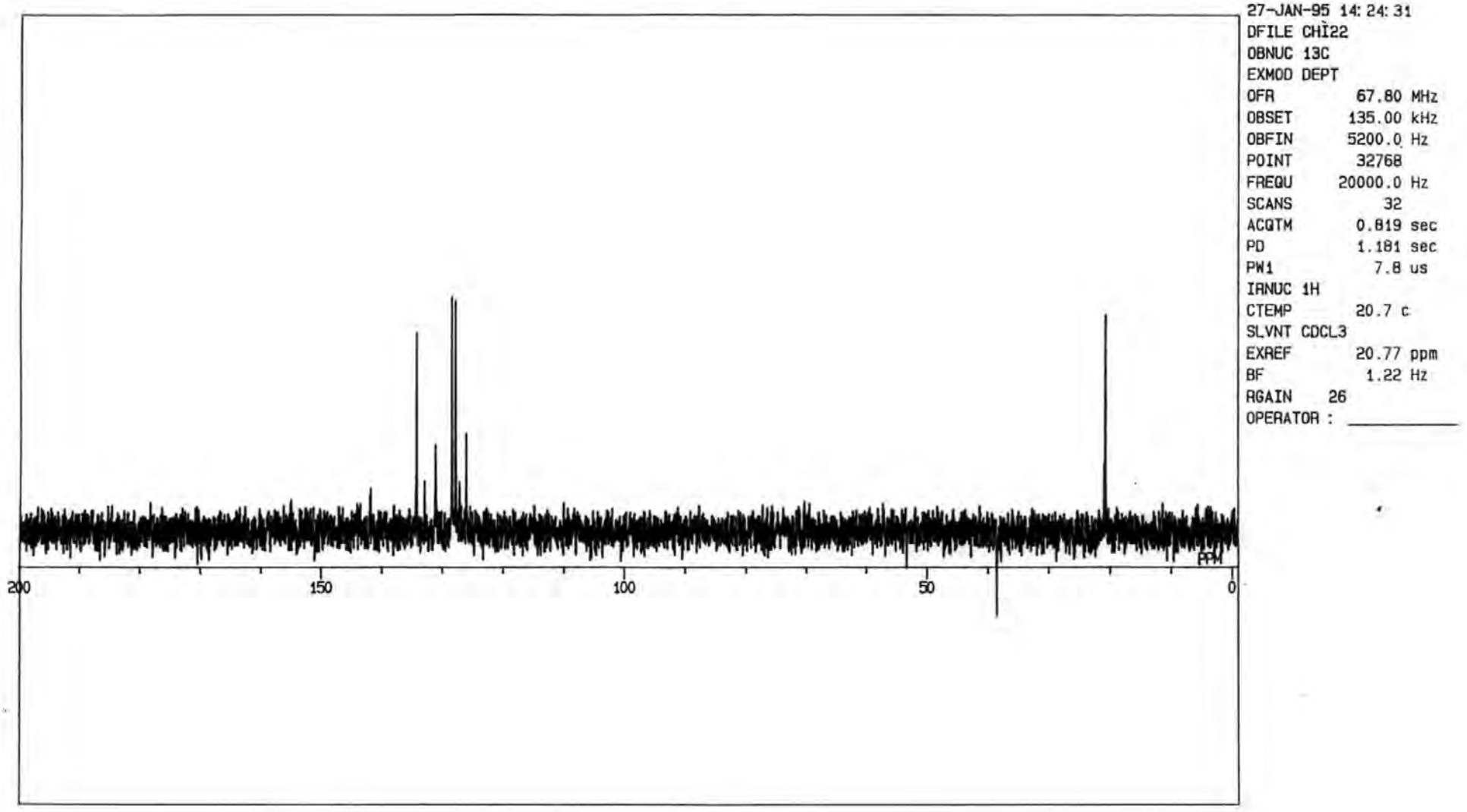
Approved ..... Managing and Technical Director



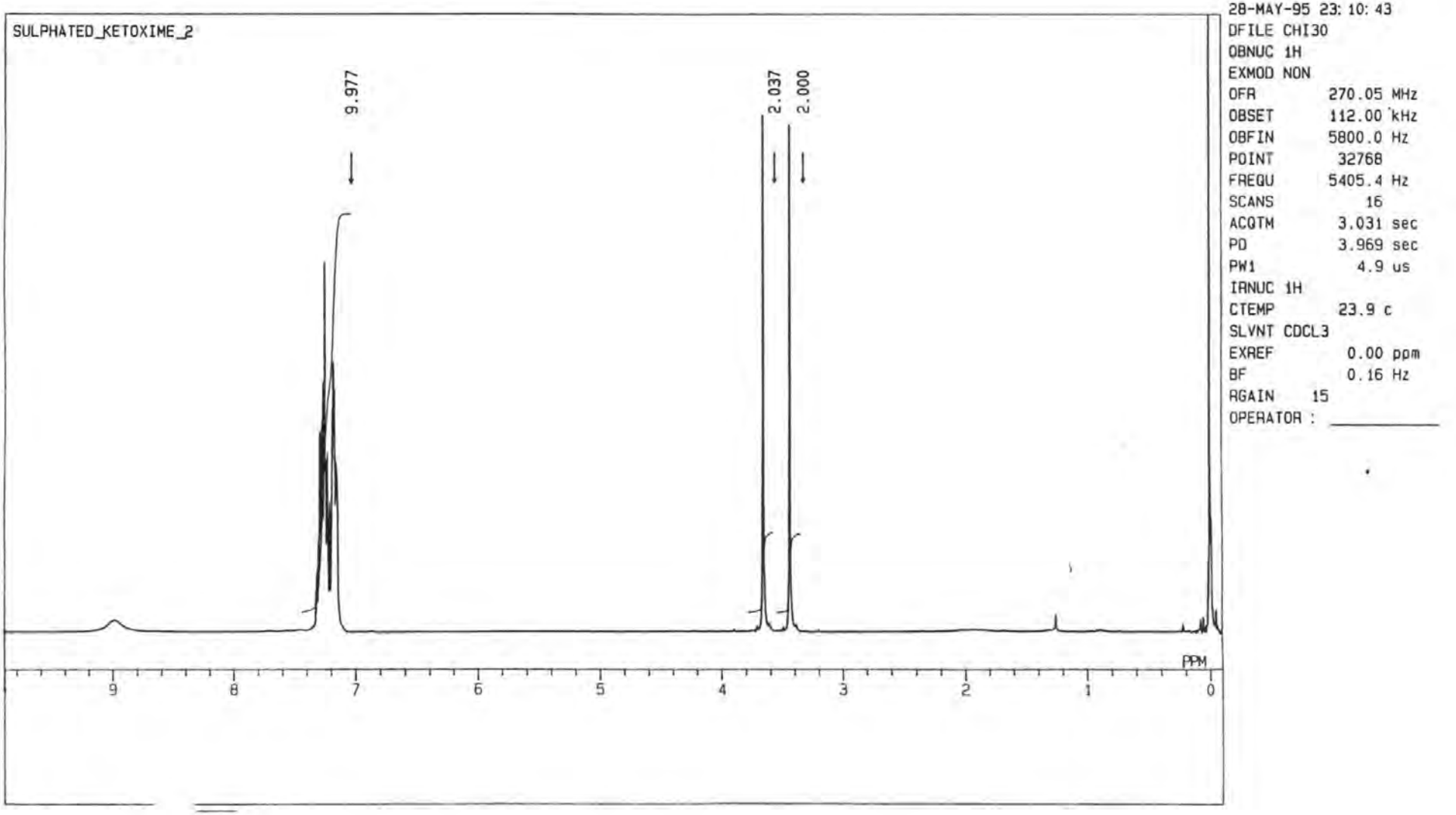
^{1}H NMR OF THE PRODUCT ISOLATED FROM THE ATTEMPTED SULPHATION OF *S*-(TETRA-*O*-ACETYL-*β*-D-GLUCOPYRANOSYL) BENZYLTHIOHYDROXIMATE



^{13}C NMR OF THE PRODUCT ISOLATED FROM THE ATTEMPTED SULPHATION OF *S*-(TETRA-*O*-ACETYL-*β*-D-GLUCOPYRANOSYL) BENZYLTHIOHYDROXIMATE



DEPT NMR OF THE PRODUCT ISOLATED FROM THE ATTEMPTED SULPHATION OF *S*-(TETRA-*O*-ACETYL-*β*-D-GLUCOPYRANOSYL) BENZYLTHIOHYDROXIMATE



^{1}H NMR OF THE PRODUCT ISOLATED FROM THE ATTEMPTED SULPHATION OF *1,3*-DIPHENYLACETONE OXIME

^{13}C NMR OF THE PRODUCT ISOLATED FROM THE ATTEMPTED SULPHATION OF *1,3*-DIPHENYLACETONE OXIME

28-MAY-95 23: 44: 17
OFILE CHI31
OBNUC 13C
EXMOD BCM
OFR 67.80 MHz
OBSET 135.00 kHz
OBFIN 5200.0 Hz
POINT 32768
FREQU 20000.0 Hz
SCANS 64
ACQTM 0.819 sec
PD 2.181 sec
PW1 3.9 us
IRNUC 1H
CTEMP 23.5 c
SLVNT CDCL3
EXREF 77.00 ppm
BF 1.22 Hz
RGAIN 26
OPERATOR : _____

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STRUCTURE–ACTIVITY RELATIONSHIPS IN GLUCOSINOLATES AND OTHER COMPOUNDS STIMULATING OVIPOSITION IN THE CABBAGE ROOT FLY (*Delia radicum*)

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Abstract—The ability of a range of glucosinolates and other compounds in stimulating oviposition of the cabbage root fly (*Delia radicum*) was determined using a bioassay based on the use of surrogate leaves coated with test compounds. The results show that chemically dissimilar compounds are effective stimulants providing they contain the S=O group. No other part of the glucosinolate molecule was shown to be necessary. It has been shown that compounds other than glucosinolates may be important in the oviposition behavior of *D. radicum*.

Key Words—Oviposition, behavior, Insecta, Diptera, Anthomyiidae, *Delia radicum*, host plant chemicals, glucosinolates, sulfate, structure–activity relationships, bioassay.

INTRODUCTION

In the cabbage root fly, *Delia radicum* (L.) (Diptera, Anthomyiidae), oviposition behavior is associated with host plant location and recognition. The host plants are found mainly within the family Cruciferae and facets of behavior are influenced by host plant chemicals that are largely specific to that family (Hawkes et al., 1978). Before contact with the plant, isothiocyanates induce upwind

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orientation and increase flight activity in mated and gravid females (Hawkes and Coaker, 1979). The behavioral outcome is the movement upwind of females in a series of flights. On landing on the host plant, glucosinolates stimulate the first of a series of behavior patterns that result in eggs being laid generally in the soil in the vicinity of the host plant (Tranyier, 1967). Contact with glucosinolates or host plant material is necessary for oviposition, the presence of volatile isothiocyanates being insufficient alone (Nair and McEwen, 1976).

Electrophysiological studies (Roessingh et al., 1992) have shown that tarsal contact chemoreceptors occur in female cabbage root fly that are sensitive to glucosinolates. The biological activity of a range of glucosinolates was investigated using electrophysiological recording and a bioassay based on the use of surrogate leaves coated with test compounds. A significant correlation was found between the overall length of the side chain of the glucosinolate molecule and biological activity. The most stimulating fraction of plant surface extract contained no glucosinolates, and it was concluded that compounds in addition to glucosinolates play an important role in oviposition.

In this paper we report the results of an investigation into the structure–activity relationship of glucosinolates and other related compounds as oviposition stimulants for *D. radicum* using a bioassay method.

METHODS AND MATERIALS

Insects. All experiments and the culture of flies were carried out in a climate controlled room (18 L:6 D-photoperiod, 21 ± 1.5°C and 70 ± 10% relative humidity). A culture was maintained using the method of Finch and Coaker (1969).

For the bioassays, emerging flies were transferred into a separate holding cage every 24 hr and used in experiments when 7–8 days old.

Oviposition Sites. The design of the surrogate leaf structure was adapted from Roessingh and Städler (1990) and Koštál (1993). Each leaf consisted of a circle of white cardboard (9 cm diameter) with vertical folds 1 cm apart. This was slotted into a plastic tube stem (4 cm length, 0.5 cm diameter). The structure was then coated with a uniform layer of paraffin wax (Merck, mp 42–44°C) by a short immersion into warm water with a floating layer (0.6–1 cm) of the molten wax on top. Three leaves were sprayed with 0.6 ml of a 2.5×10^{-5} M solution of sinigrin in 75% aqueous methanol as the standards, three were treated with 0.6 ml of a test solution at a known concentration in 75% aqueous methanol, and the final three were treated with 75% aqueous methanol as the blanks. The model leaves were left to dry for approximately 30 min and then each one was fixed into the center of a Petri dish (9 cm diameter, 1.5 cm deep). Fine sand was placed into the dish around the leaf in which the flies could lay their eggs.

TEST 1	TEST 2	BLANK
BLANK	TEST 1	TEST 2
TEST 2	BLANK	TEST 1

FIG. 1. Layout of surrogate leaf structures on the bioassay cage floor. Test 1 is the control standard of leaves treated with 2.5×10^{-5} M sinigrin solution and test 2 is the compound under test.

The accuracy of the application technique in the preparation of the surrogate leaves was tested by recovery experiments using HPLC (Spinks et al., 1984). Sinigrin was recovered 95% or greater levels from the leaves in four repeat experiments.

Bioassays. Bioassay experiments were performed in screen cages (50 × 50 × 50 cm), on the floor of which were placed nine surrogate leaf structures in a random grid arrangement (Figure 1). Ten female flies, 7–8 days old, and an average weight of 11 mg, were introduced into the cage for 24 hr, after which times the "leaves" were removed and the number of eggs laid on and around each one determined by flotation in water.

Treatment of Results. The best-fit model of the observed data was found to be a cubic equation relating the fly oviposition response to the natural log of the concentration as shown:

$$Y = ax^3 + bx^2 + cx + d$$

where x is $\log_{10}$ concentration (molar), Y is relative stimulation $(T_2 - B)/(T_1 - B)$, with T_1 the total number of eggs laid on the sinigrin control standards (test 1), T_2 the total number of eggs laid on the "leaves" treated with the test compound (test 2), and B the total number of eggs laid on the blanks.

The optimum concentration (C) of a compound required to give maximum relative stimulation (Y_{max}) for each compound was then calculated from the model and used as a measure of biological activity. Compounds were designated as biologically active if egg laying on the treated sites was significantly higher than that on the blanks according to the F test for variance and the appropriate t test. A value of Y greater than 0.01 was obtained as the level indicating significant oviposition stimulation.

Test Compounds and Their Synthesis. All test compounds and the starting materials were purchased from Aldrich UK Ltd. and used as received unless stated otherwise. Potassium salts of three different glucosinolates were used: sinigrin, glucobrassicin, and glucotropaeolin. Sinigrin was purchased from Sigma UK Ltd., and the other two were provided by the Department of Biological

Sciences, University of Plymouth. Desulfosinigrin, sinigrin tetraacetate, acetone sodium bisulfite, *S*-methyl cysteine sulfoxide, and the corresponding sulfone were synthesized as described below.

Desulfosinigrin was obtained by the desulfation of the glucosinolate molecule using the enzyme aryl sulfatase (Sigma UK Ltd.). The enzyme (10 mg) was added to 5 mg of sinigrin dissolved in 50 ml of 0.2 M acetate buffer, pH 5.57. The solution was left overnight at 25°C and the desulfation was considered to be complete since the sample was chromatographically homogeneous following the procedure of Spinks et al. (1984) with no trace of sinigrin present. The reaction solution was bioassayed immediately with an appropriate blank.

Sinigrin tetraacetate was synthesized according to the method of Kjaer and Gmelin (1956). A 10-fold excess of acetic anhydride was used, and the reaction mixture was kept at 50°C for 6 hr. The product had a melting point of 194°C (lit. value 194°C; Benn and Ettlinger, 1965). The material was chromatographically homogeneous as shown by thin-layer chromatography. R_f values of 0.27 and 0.6 were obtained on 60 G silica gel plates for sinigrin and its tetraacetate, respectively, with an *n*-butanol–acetic acid–water (60:15:25% by volume) solvent system.

The bisulfite addition compound was prepared according to Vogel (1970) and *S*-methyl cysteine sulfoxide according to Greenstein and Winitz (1961). *S*-Methyl cysteine sulfone was synthesised by the oxidation of *S*-methyl cysteine sulfoxide.

RESULTS

Table 1 shows the structures, optimum concentrations (C), and maximum stimulation relative to the 2.5×10^{-5} M sinigrin standard (Y_{max}) for three naturally occurring glucosinolates and two structural modifications of sinigrin. Table 2 shows similar data for 12 other nonglucosinolate compounds tested, plus one mixture.

Figures 2–4 show plots of relative stimulation (Y) versus $\log_{10}$ concentration (x) for all of the compounds tested together with the best-fit curves in each case from which C and Y_{max} can be calculated.

Glucobrassicin (Table 1, compound 1) proved to be the most effective compound in terms of number of eggs laid relative to a standard (Y_{max}). Examination of Tables 1 and 2 together with Figures 2–4 shows that the concentration of a compound (C) required to produce the maximum stimulus (Y_{max}) shows a considerable range (4.29×10^{-4} to 1.16×10^{-6} M). All of the bioactive compounds show a decrease in activity at higher concentrations.

Figure 5 shows a plot of maximum relative stimulation (Y_{max}) against optimum concentration (C) for each compound. With the exception of sinigrin and

TABLE 1. NAMES, STRUCTURES, AND DETAILS OF ACTIVITY OF NATURAL AND MODIFIED GLUCOSINOLATES BIOASSAYED

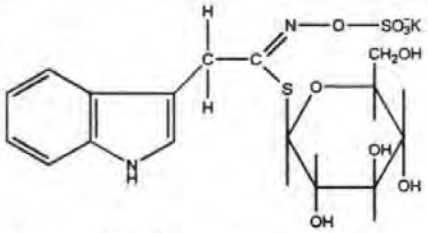
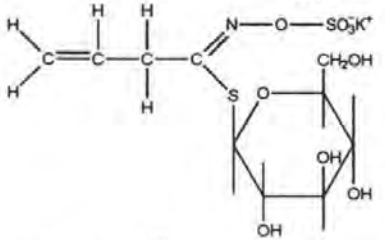
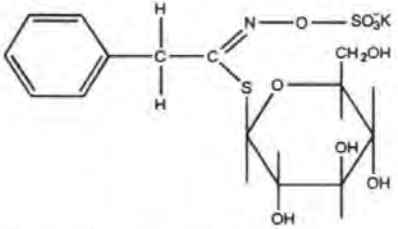
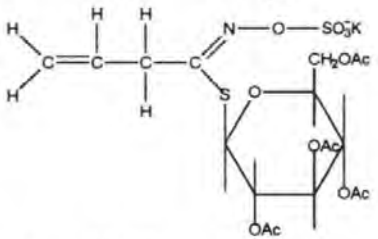
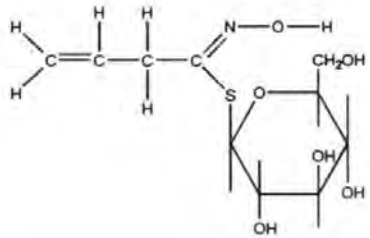
No.	Structure	Compound name	C	Y_{max}
1		Glucobrassicin	1.4×10^{-5}	5.30
2		Sinigrin	3.8×10^{-4}	2.82
3		Glucotropaeolin	2.2×10^{-6}	1.83
4		Sinigrin tetraacetate	5.5×10^{-5}	1.17
5		Desulfated sinigrin	Not active	Not active

TABLE 2. NAMES, STRUCTURES, AND DETAILS OF ACTIVITY OF NONGLUCOSINOLATE TEST COMPOUNDS BIOASSAYED

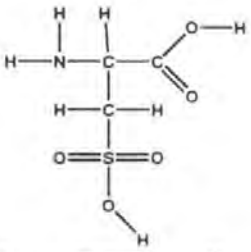
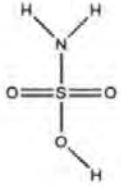
No.	Structure	Compound name	C	Y_{max}
1		Cysteic acid	1.97×10^{-6}	1.57
2		*p*-Toluene sulfonic acid	8.36×10^{-5}	1.10
3		Taurine	1.16×10^{-6}	1.63
4		Hydroxylamine-*O*-sulfonic acid	4.39×10^{-5}	1.67
5		Sulfamic acid	4.99×10^{-5}	2.01

TABLE 2. CONTINUED

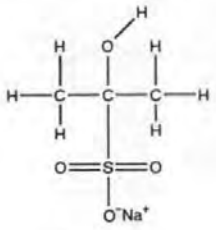
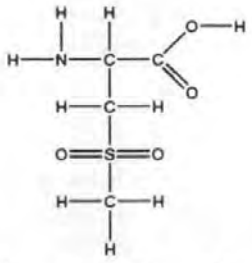
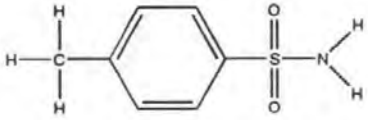
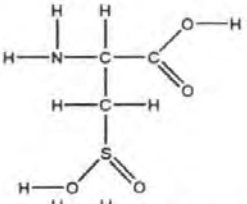
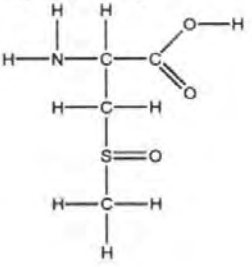
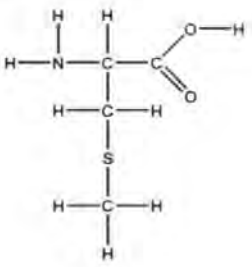
No.	Structure	Compound name	C	Y_{max}
6		Acetone sodium bisulfite	4.29×10^{-4}	0.7
7		*S*-Methyl cysteine sulfone	4.11×10^{-6}	1.37
8		*p*-Toluene sulfonamide	1.37×10^{-6}	1.12
9		Cysteine sulfinic acid	1.59×10^{-6}	1.28
10		*S*-Methyl cysteine sulfoxide	1.44×10^{-6}	1.16

TABLE 2. CONTINUED

No.	Structure	Compound name	C	Y_{max}
11		*S*-Methyl cysteine	Not active	Not active
12	$NaHSO_4$	Sodium bisulfate	Not active	Not active

glucobrassicin, all the bioactive compounds lie in a narrow range of Y_{max} values (between 1 and 2). Compounds with the low optimum concentrations have high biological activity but the high relative stimulation of glucobrassicin also indicates high activity.

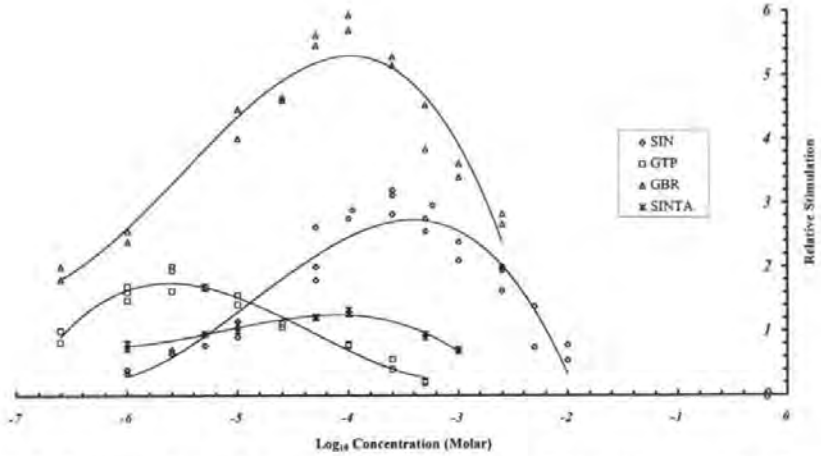


FIG. 2. Bioassay data and regression lines for sinigrin (SIN), glucotropaeolin (GTP), glucobrassicin (GBR), and sinigrin tetraacetate (SINTA).

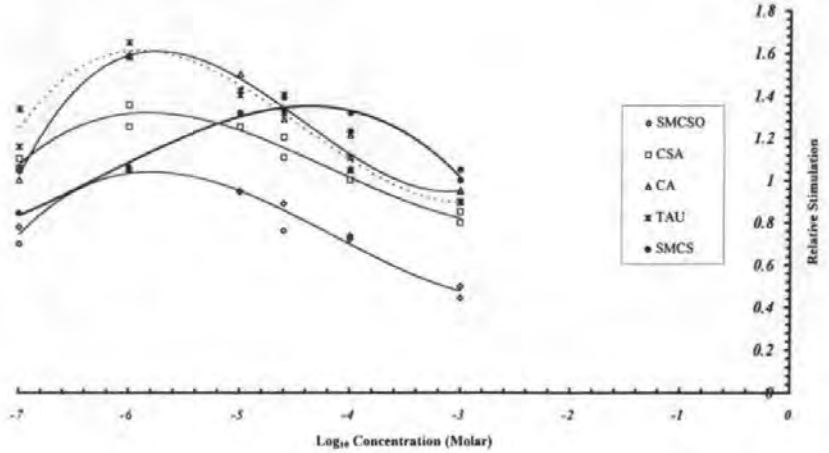


FIG. 3. Bioassay data and regression lines for *S*-methyl cysteine sulfoxide (SMCSO), cysteine sulfinic acid (CSA), cysteic acid (CA), taurine (TAU), and *S*-methyl cysteine sulfone (SMCS).

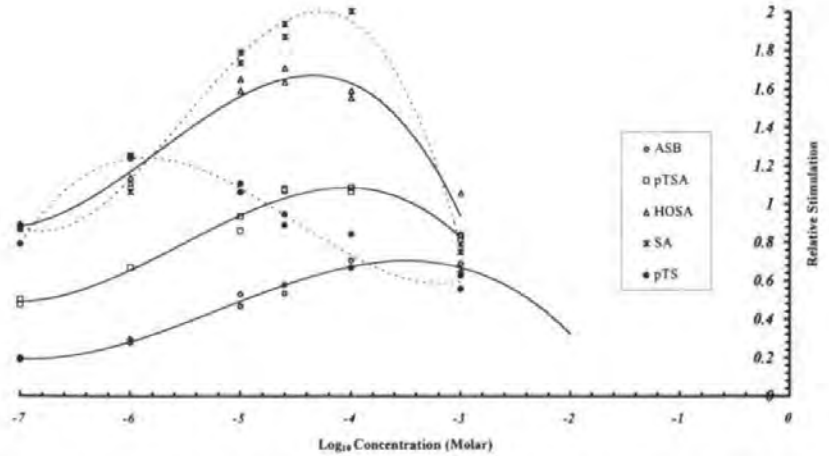


FIG. 4. Bioassay data and regression lines for acetone sodium bisulfite (ASB), *p*-toluene sulfonic acid (pTSA), hydroxylamine-*O*-sulfonic acid (HOSA), and *p*-toluene sulfonamide (pTS).

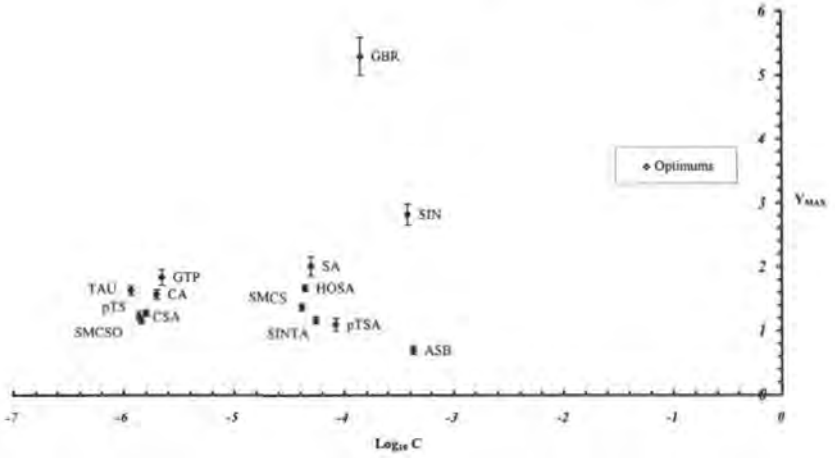


FIG. 5. Optimum concentrations and responses for all active test compounds.

DISCUSSION

The naturally occurring glucosinolate molecules can be divided into three structural regions: a side-chain (R), a thioglucose, and a sulfated oxime. Examination of Table 1 shows that variations in R result in modification of activity, demonstrated by sinigrin, glucobrassicin, and glucotropaeolin. Similar findings have been reported by Roessingh et al. (1992) for *D. radicum* and by Simmonds et al. (1994) for *D. floralis*.

Modification of the thioglucose residue of sinigrin by acetylating all of the hydroxyl groups to give sinigrin tetraacetate caused some decrease in activity. The level was well above the threshold of nonstimulus, however. This implies that the glucose residue is not a key structural requirement.

Desulfation of the oxime sulfate group resulted in the formation of desulfated sinigrin, which was devoid of all activity. The sulfate group seems to be an essential structural feature for oviposition—a region that could be identified as an on–off switch for egg laying.

A series of compounds containing the SO_3H group bonded to a variety of other organic residues or functional groups was then examined to test this hypothesis. Table 2, compounds 1–6, shows that all the compounds examined that contained the SO_3H group possessed significant biological activity despite the wide variety of structures attached to the group. Toluene-*p*-sulfonic acid (2) was tested as both a free sulfonic acid and as the potassium salt. There was no difference in the bioassay results, showing that it is not necessary to have the ionized SO_3^- group rather than the parent acid. The most effective compound

in terms of Y_{max} was the simple compound sulfamic acid (5), in which the sulfur atom is bonded directly to an amino-nitrogen atom. Hydroxylamine-*O*-sulfonic acid (4), which contains the three atom system N-O-S present in glucosinolates, was also an effective stimulant.

Attempts to sulfate the oxime group of simple ketones to produce compounds more strictly analogous to glucosinolates were unsuccessful, the reaction producing an amide by a spontaneous Beckmann rearrangement of the desired product. This result was not unexpected.

The results obtained from compounds showing a progressive decrease in the oxidation state of the original SO_3H sulfur atom are given in Table 2, compounds 6–11. From these results it becomes quite clear that the key structural requirement for oviposition stimulus is the presence of an S=O group in a molecule. This group is present in *S*-methyl cysteine sulfoxide (10), which was bioactive. *S*-Methyl cysteine, lacking the S=O group, was devoid of biological activity. Sodium bisulfate (12), in which the S atom is in its maximum oxidation state, also lacked activity.

Städler and Shöni (1990) produced evidence that more than one group of compounds stimulates oviposition. Roessingh et al. (1992) report that compounds additional to glucosinolates play a role in the stimulation of oviposition. The compounds were not identified, however.

S-Methyl cysteine sulfoxide (10) is known to be present in the leaves of a variety of Brassicae, including cabbage at significant levels (Morris and Thompson, 1956; Synge and Wood, 1956). In view of the demonstration of its oviposition-stimulating ability on surrogate leaves, it is possible that it could have the same property in vivo. However there was no synergistic activity between sinigrin and (10) (Table 3), the effect being only additive.

TABLE 3. RESULTS OF SYNERGISM EXPERIMENTS WITH SINIGRIN AND *S*-METHYL CYSTEINE SULFOXIDE

Compounds	Initial concentration (M)	% Used in test mixture	Concentration in test mixture (M)	Expected *Y* (additive)	Actual *Y*
Sinigrin +	1×10^{-5}	90	9×10^{-6}	2.31	2.00
S-methyl cysteine sulfoxide	1×10^{-4}	10	1×10^{-5}		
Sinigrin +	1×10^{-4}	50	5×10^{-6}	1.78	1.72
S-methyl cysteine sulfoxide	1×10^{-4}	50	5×10^{-5}		
Sinigrin +	1×10^{-5}	10	1×10^{-6}	1.05	0.98
S-Methyl cysteine sulfoxide	1×10^{-4}	90	9×10^{-5}		

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