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Developing a fungicide, azoxystrobin

The world wide market for agricultural fungicides (compounds used to treat fungal diseases in plants) is around £4 billion per year. This study looks at the development by Zeneca of a new plant fungicide, azoxystrobin, formerly code named ICIA5504 , and the processes which it has to go through from the initial discovery to being on sale – a process that can take up to 15 years. Incidentally the code name reflects the fact that the development of ICIA5504 began before the de-merger of ICI and Zeneca in 1993. The story is typical of the development of other agrochemicals (chemicals used by farmers to treat crops). *Figure 1* summarises the main parts of that process diagramatically.

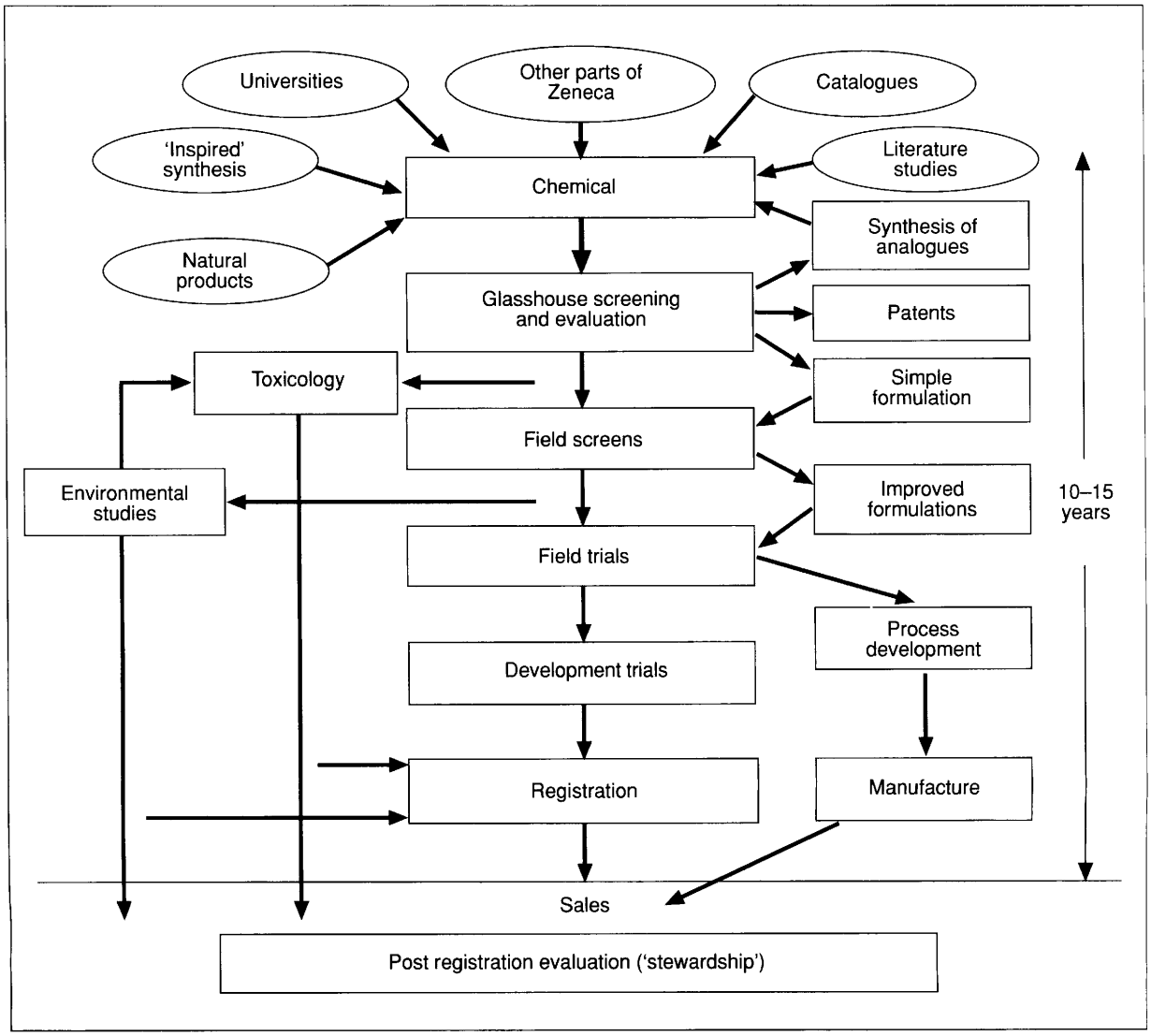

Figure 1 The discovery and development of a new pesticide

Table 1 Many people from several disciplines are involved over a period of years in the development of an agrochemical such as azoxystrobin

Year	1	2	3	4	5	6	7	8	9
Research chemist	Selection of compound and small scale synthesis (100 g)								PRODUCT LAUNCH
Economist		Feasibility studies				Choice of manufacturing site		Construction of plant	
Toxicologist	Acute toxicology tests		Studies on production samples						
Environmental scientist			Determination of fate in the environment. Preliminary studies on ecotoxicity →				→		
Biologist	Screening for activity		Tests in the field →				→		
Patent officer	Patent application						More patent applications		
Process development chemist		Pilot plant for larger scale production (100 kg) for testing		Development of large scale synthesis →			→		
Registration officer					Application for registration				
Formulation chemist				How to apply the required dose?	Stability tests. Packaging				
Marketing specialist		Compare with current market leader or is this a new market?				How and where to launch the product?			

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Development of a fungicide or other agrochemical is carried out by multidisciplinary teams. Table 1 shows some of the disciplines involved, what each one does and when, although details and time scale will vary from product to product. In this study we look at the discovery and environmental testing of azoxystrobin.

Discovery

As indicated in *Fig 1*, the initial discovery of compounds with particular types of activity comes from a variety of sources. In a typical year, Zeneca might test 100 000 compounds for activity of which fewer than 500 might be selected for further testing and 1 or 2 might eventually be developed.

In the case of azoxystrobin, a family of natural products, the strobilurins, oudemansins and myxothiazols (all related in structure to β-methoxyacrylic acid – structures 1–4) were found to have fungicidal activity. This was noted in 1981 when an ICI researcher read an account of them in a German research paper.

Note. β-methoxyacrylic acid's systematic name is 3-methoxypropenoic acid but the systematic names of the other compounds are too long for everyday use. In this case study we shall use the trivial names by which they are normally known.

Despite having fungicidal activity, these compounds were found in fungi growing on decayed wood. It is not yet clear how it is that these fungi are not affected by the fungicidal compounds they themselves synthesise. However, the fungicides seem to help the parent organism to compete with other fungi. The fungicidal compounds have been found to work by inhibiting electron transport within the cells of fungi.

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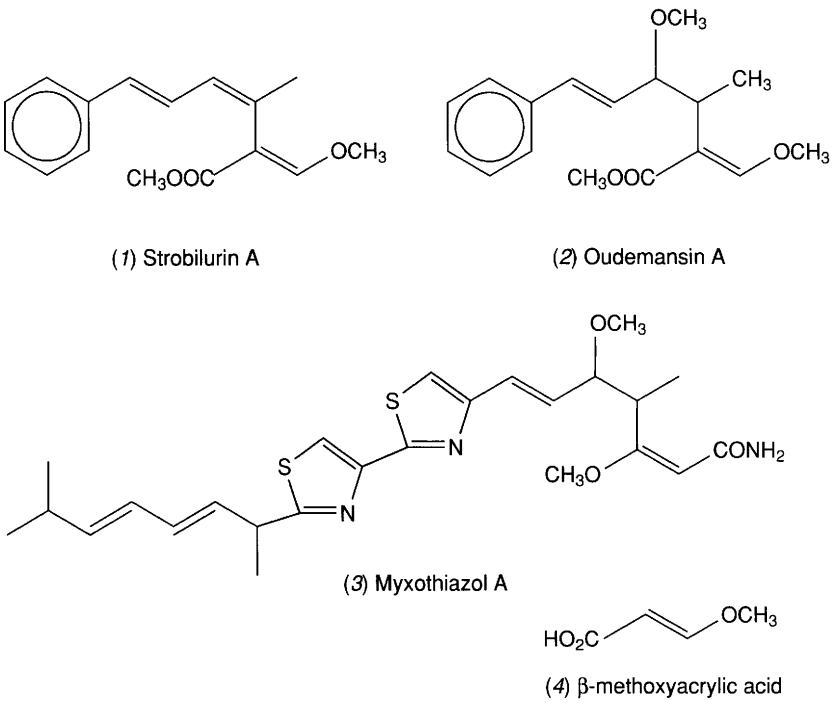

Fig 2 β-Methoxyacrylic acid and related compounds

Initially the fungicidal activity of these natural products was confirmed by glasshouse tests.

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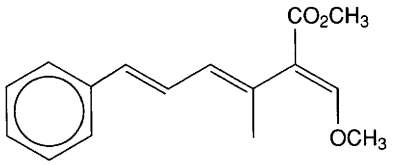
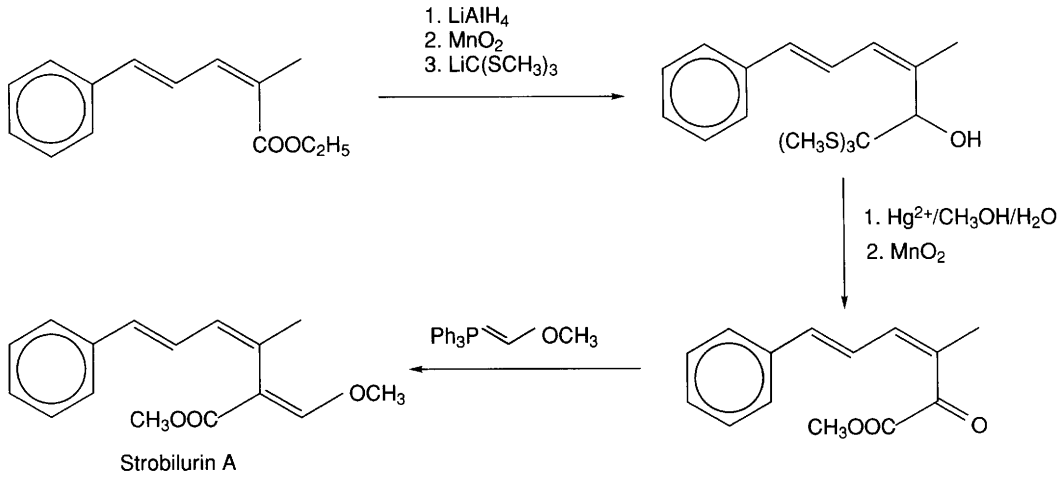
A structural problem **Box 1**

The simplest compound of the series was strobilurin A and it was considered important to establish its fungicidal activity. To do so, a method of making it in the laboratory had to be devised. The structure of strobilurin A had been reported in the literature as (*5*). Two syntheses were devised to make the compound with this structure but it was found not to be the same as strobilurin A. Careful analysis of the spectroscopic data indicated that strobilurin A had the structure (*1*) and a synthesis was devised which led to the correct compound – see below.



(*5*) The wrongly-reported structure of strobilurin A



The synthesis of strobilurin A used to confirm its structure

Question 1

Look at the structure of strobilurin A and also the proposed but incorrect one. Can you see how they are related? Making models might help if you have access to a molecular modelling kit.

Strobilurin A (*1*) which is structurally the simplest compound, proved relatively ineffective in glasshouse trials as it broke down quickly in light and was also quite volatile. This meant that it did not remain on the leaf for long after spraying. It did, however, show activity against fungi growing on agar in petri dishes in low light conditions.

So a number of derivatives (related compounds) of strobilurin A were synthesised. These retained the β-methoxyacrylate unit which was believed to be responsible for the fungicidal activity of the compounds. These included compounds (*6*), (*7*), and (*8*) (*Fig 3*).

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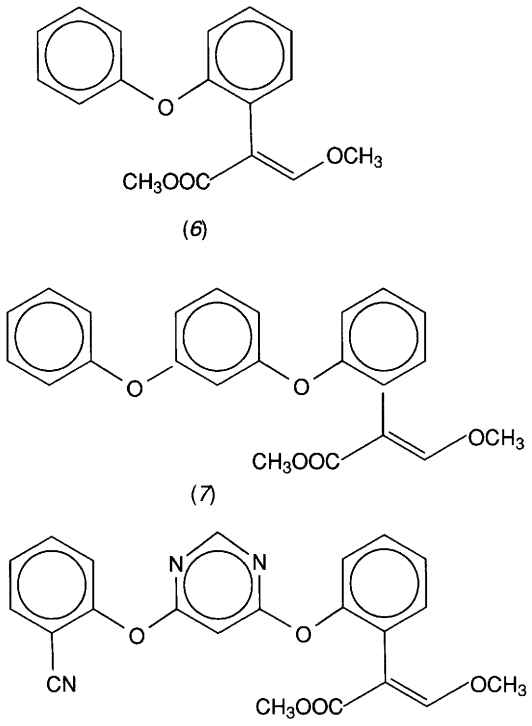

Figure 3 Compounds synthesised during the development of azoxystrobin

Compound (*6*), containing a diphenyl ether unit turned out to be less volatile and more photochemically stable than strobilurin A. It moved systemically through the plant, *ie* it was transported through the plant tissue – an important advantage. It showed good fungicidal activity but it also damaged the plants in trials.

Addition of a further aromatic ring gave compound (*7*), which had even better fungicidal activity but was too insoluble in water to move systemically.

Incorporation of electronegative nitrogen atoms to form a heterocyclic aromatic ring improved the water solubility and eventually azoxystrobin (*8*) was produced. This had a solubility in water of around 10 mg dm^{-3}, and, in a laboratory test, it took 20–35 hours for half of it to decay in light equivalent to a bright summer's day. It was effective against a range of fungi. In particular, it controlled both of the two key fungi which affect vines and rice. Previously, two different fungicides had to be applied to control these.

Question 2

Can you explain why incorporating nitrogen atoms into the molecule improved its water solubility? What type of intermolecular forces are involved?

A further important point is that azoxystrobin has a relatively low toxicity. Toxicity is expressed as an LD_{50} (lethal dose, 50%) – the dose (in grams of test substance per kilogram of body mass) required to kill half of a population of test animals. The *lower* the LD_{50}, the *more* toxic the substance. Azoxystrobin's LD_{50} for oral administration to rats is 5 g kg^{-1}. For comparison, the LD_{50} of aspirin is 1.5 g kg^{-1} and the LD_{50} of caffeine is 0.13 g kg^{-1}. So azoxystrobin is less toxic than either of these everyday compounds.

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At this stage, the Zeneca management had to take a decision whether to proceed with azoxystrobin or not. To proceed would mean spending several million pounds on toxicity and environmental tests and, at the same time, devising a suitable method to make it on a large scale. These projects had to run in parallel for time reasons – to run them one after the other would lead to an unacceptable delay in getting the product on the market. However, this involved the risk that spending on the synthetic method would be wasted if the compound failed in the other trials.

Toxicology

Preliminary studies must be done to measure the toxicity of a compound (when ingested by mouth, through the skin or by inhalation) for legal reasons and so that appropriate precautions can be taken for use, handling and transport during development. A compound might be discarded at this stage because it could be too toxic to develop and use. There is also an ethical decision to be made – should Zeneca sell such a compound? Later on, toxicology tests have to be repeated using material made by the production process as different methods of synthesis might generate different impurities which may themselves be toxic.

Environmental fate and effects

For all agrochemicals, two key questions must be answered before the substance can be registered and sold.

1. What happens to the chemical after it has been applied?
2. What effect does the chemical (and its breakdown products) have on living things (plants and animals) exposed to it?

Question 1 relates largely to the chemistry of the compound – its solubility and stability. It involves the rate of breakdown when exposed to soil, water, light and in plants and animals. It also involves identifying the breakdown products and their properties, *ie* toxicity, water solubility, do they bind to soil? Measurements are made of the concentration of the compound and its breakdown products (called "degradates", or "metabolites" if they are the result of biological degradation) in soil, water, the crop itself and other plants and animals which might be exposed to it in a variety of ways. This is done first in laboratory or glasshouse trials and then by trials in the field – usually in a number of different places across the world and over a number of years so that a variety of different conditions is experienced. At Zeneca, samples of plant material, soil and water are brought to the Jealott's Hill Research Station in Berkshire for measurements of residue levels.

Question 2 relates to the biological effects of the compound and its breakdown products and their effects on a variety of living things from microbes to mammals.

Chemical testing

Much of the chemical testing is concerned with measuring levels of residues of the applied chemical and its degradates and metabolites in a variety of samples, routinely down to levels of 0.01 part per million (ppm). This is done by grinding the samples, extracting the residues into a suitable solvent and separating them by gas chromatography (GC) or high performance liquid chromatography (HPLC) with a suitable detector, often a mass spectrometer.

Note. A level of 0.01 ppm is equivalent to finding one sheet of paper in a pile of paper the height of Mount Everest.

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High performance liquid chromatography (HPLC) **Box 2**

High performance liquid chromatography (HPLC) is one of the most important modern chromatographic separation techniques. A solvent mixture (the mobile phase) is forced at high pressure through a powdered solid stationary phase (often silica). The mixture to be separated is injected into the mobile phase and is separated on the column. The detector is often a mass spectrometer which can identify components from their relative molecular masses and fragmentation patterns. Very efficient separations are possible with HPLC – even pairs of optical isomers can be distinguished with an appropriate stationary phase.

As well as chemical testing, biological tests are carried out on the effects of agrochemicals on non-target plants, soil micro-organisms, earthworms, insects, birds, fish, aquatic invertebrates, algae and mammals both in the field and the laboratory. Non-target plants are those which might be treated by accident (by spray drift, for example) as opposed to the crop to which the fungicide is deliberately being applied.

Studies include acute tests (which measure lethality) and chronic (long term) studies which measure effects on growth and reproduction of organisms exposed to the test chemical. The potential of the test chemical to accumulate in the food chain is also measured.

For example, tests of the effect of azoxystrobin on the aquatic environment included acute and chronic studies on fish species (including trout, carp and bluegill sunfish), invertebrates (such as *Daphnia*) and algae. The concentrations at which effects were observed were then compared with the concentrations which were predicted to occur in the environment, to see if adverse effects were likely. Toxicity tests were also carried out on a variety of invertebrates including insects, snails, worms and zooplankton. Bioconcentration factors may also be measured for some products. These are indications of how much a substance is concentrated in different organisms. For example, a fish might be found to have 100 times the concentration of a test chemical in its body compared with the water in which it was living.

Tests were carried out in both laboratory and field and the results indicated that azoxystrobin would pose a negligible risk to the aquatic environment.

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Assessing risk **Box 3**

Much of the development work on agrochemicals is involved with the assessment of the risk involved in exposure to certain chemicals. The risk of exposure is related to two factors:

- the inherent toxicity of the chemical itself which can be expressed by its LD_{50}; and
- the level of exposure to the chemical.

So even a highly toxic compound presents very little risk if the exposure is very low and conversely even a relatively innocuous chemical, such as caffeine, can kill if the dose is high enough.

For residues in the diet, an Acceptable Daily Intake (ADI) is used to estimate the amount of a residue that can be safely consumed over a whole lifetime. The ADI is derived from feeding trials on a variety of laboratory animals. These enable a no observed effect level (NOEL) to be found for the species which is most sensitive to a particular chemical. An uncertainty factor of 100 (1000 for chemicals which have shown any evidence of causing cancer) is then built in to give an ADI which is measured in mg of chemical per kg of body mass per day.

Radiolabelling

Radiolabelling can be useful in studying where the compound and its breakdown products appear in the environment. The pesticide can be synthesised so that radioactive atoms (usually ^{14}C) are incorporated in it. ^{14}C is a β-emitter so the fate of the compound and any of its breakdown products which contain ^{14}C can be traced by measuring the radioactivity emitted. That is, the location of the activity can be traced even if the chemical identity of the product is unknown. Radiolabelled compounds are expensive to prepare so once the radioactivity has been located and the compound it is in has been identified, further work can proceed using unlabelled material. ^{14}C-containing compounds can also be detected by mass spectrometry because a fragment ion containing a ^{14}C atom has a mass two units greater than one containing ^{12}C.

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Synthesising ^{14}C labelled compounds **Box 4**

^{14}C is made in a nuclear reactor by bombarding ^{14}N containing-compounds with neutrons. This is followed by ejection of a proton from the nitrogen nucleus to form ^{14}C.

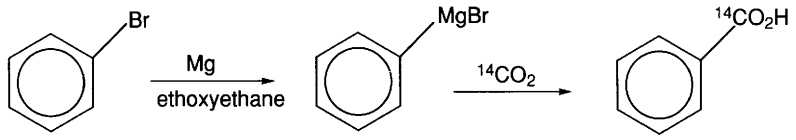
$$^{14}_{7}N + {}^{1}_{0}n \rightarrow {}^{14}_{6}C + {}^{1}_{1}p$$

The resulting $^{14}_{6}C$ decays back to $^{14}_{7}N$ by β-emission with a half-life of 5730 years. This long half-life means that there will be no significant loss of radioactivity caused by radioactive decay during the time scale of the experiments (less than 10 years).

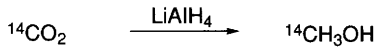
The compounds which are bombarded with neutrons are beryllium nitride (Be_3N_2) or aluminium nitride (AlN), chosen for their high nitrogen content, stability to heat and radiation and lack of contamination with ^{12}C. The resulting beryllium or aluminium carbides are oxidised to give $^{14}CO_2$ and reacted with barium hydroxide to give barium carbonate ($Ba^{14}CO_3$) from which $^{14}CO_2$ can be produced. This is the starting material for all ^{14}C-labelled organic compounds.

The labelled carbon can be incorporated into organic compounds in a variety of ways.

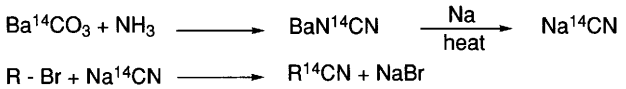
1. Carboxylation via a Grignard reaction to give carboxylic acids labelled at the carboxyl carbon, for example:



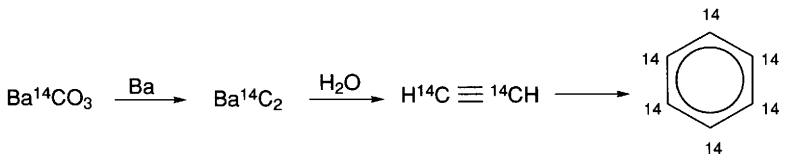
2. Reduction to methanol by lithium aluminium hydride (lithium tetrahydridoaluminate(III)), for example:



3. Reaction with ammonia followed by reduction to sodium cyanide which can then be converted into nitriles:



4. Reduction to barium carbide which can then be hydrolysed to give ethyne which can in turn be used to make benzene and hence a variety of aromatic compounds:



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Question 3

How would you attempt to make the following ^{14}C isotopically labelled compounds?

a. Methyl benzoate labelled on (i) the aromatic ring, (ii) the carboxyl carbon, (iii) the methyl group.

b. Propanoic acid with the label on the carboxyl carbon.

c. Phenylamine (aniline) with the ring labelled.

Radiolabelling trials have to be small scale for economic reasons because the labelled compounds are expensive to synthesise. Typically a trial might be done on a 1 m^2 plot of crop and require 25 mg of radiolabelled compound costing over £1000. A typical trial with a radiolabelled compound might go as follows (*Fig 4*).

The labelled test compound is sprayed onto a crop which is then harvested, say one month later. The crop is ground up and shaken with an appropriate solvent in which the radiolabelled compounds are soluble. A portion of this is placed in a scintillation counter to determine the total radioactivity. The radioactive compounds are then partitioned between an aqueous and an organic solvent. The compounds in each layer are next separated by thin-layer chromatography (TLC) or high performance liquid chromatography (HPLC) to determine the number of breakdown products which contain radioactivity. The soil on which the crop has grown is treated in the same way. In the laboratory, air from above the growing crop may also be sampled to determine how much of the labelled carbon has degraded to carbon dioxide. In this way the fate of the original compound can be traced.

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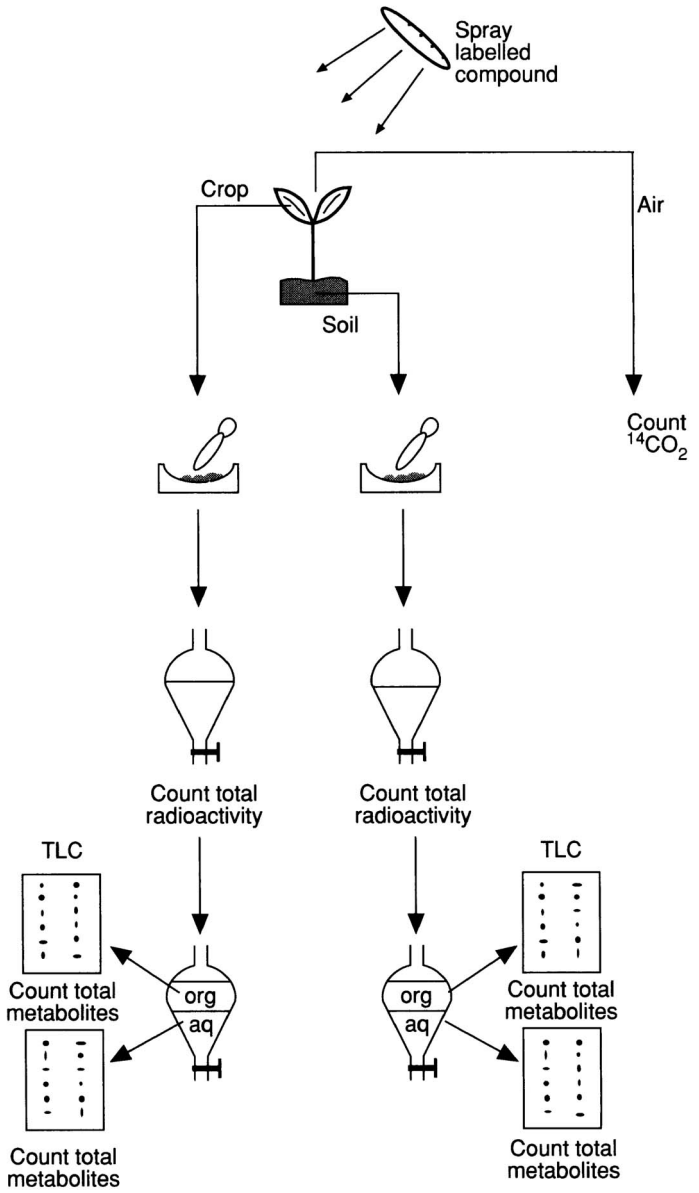

Figure 4 A radiolabelling trial

Interpreting information from radiolabelling

There are a number of issues to be considered when deciding where to label a compound. Take methyl benzoate, as a simple example. This could be labelled in the aromatic ring, the carboxylate carbon or the carbon of the methyl group (*Fig 5*).

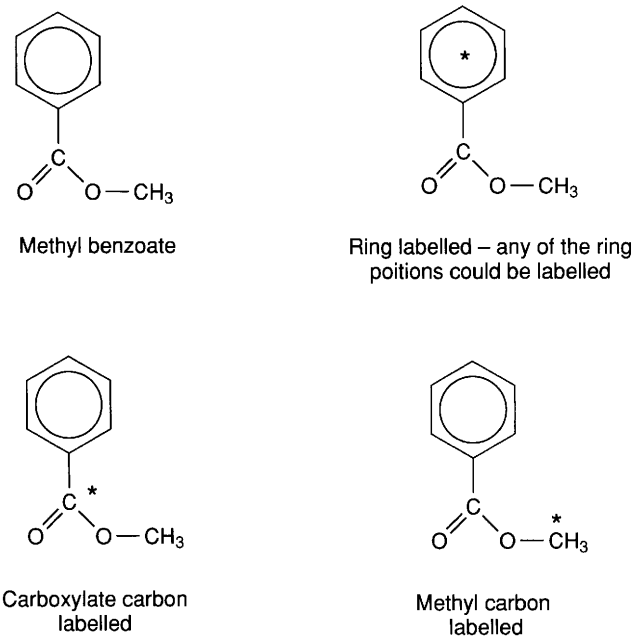

Figure 5 Possible labelling positions for methyl benzoate

However, labelling in this last position, the methyl group, would be of limited value in following the environmental fate of this compound as the methyl group will be easily lost by hydrolysis (quite likely in most environments).

Question 4

Write an equation for the hydrolysis of methyl benzoate in which the methyl group is lost. Under what sort of conditions would this take place?

So labelling in a more stable part of the molecule (such as the aromatic ring) is preferred by regulatory agencies. It is common to label more than one part of a molecule as is shown in the following (hypothetical) example with compound X (*Fig 6*).

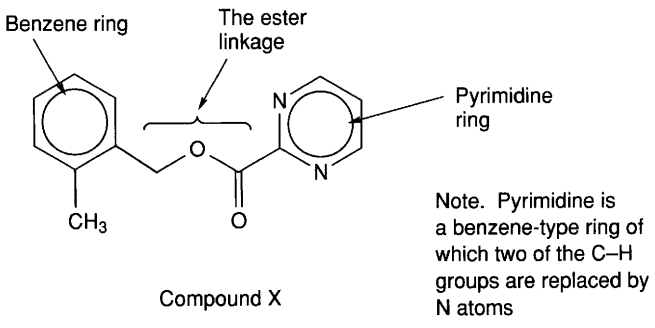

Figure 6 Radiolabelling of compound X

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Typically two radioactively-labelled samples of X would be synthesised, one with the label in the benzene ring and the other with the label in the pyrimidine ring. Let us call the benzene ring labelled compound B and the pyrimidine ring-labelled compound P. Two tests are then run, one treating a test system (crop, soil, water *etc*) with B and another an identical test system with P. Metabolites (compounds produced from X) M_1, M_2, M_3, and M_4 are separated by, say, thin layer chromatography. This is a technique similar to paper chromatography where the paper is replaced by a thin layer of a suitable stationary phase coated onto a glass or plastic backing. The β-radioactivity from the ^{14}C labels is counted by a technique known as autoradiography in which the chromatogram is sandwiched next to an imaging plate which is sensitive to radioactivity. A typical result might be as shown in *Fig 7*.

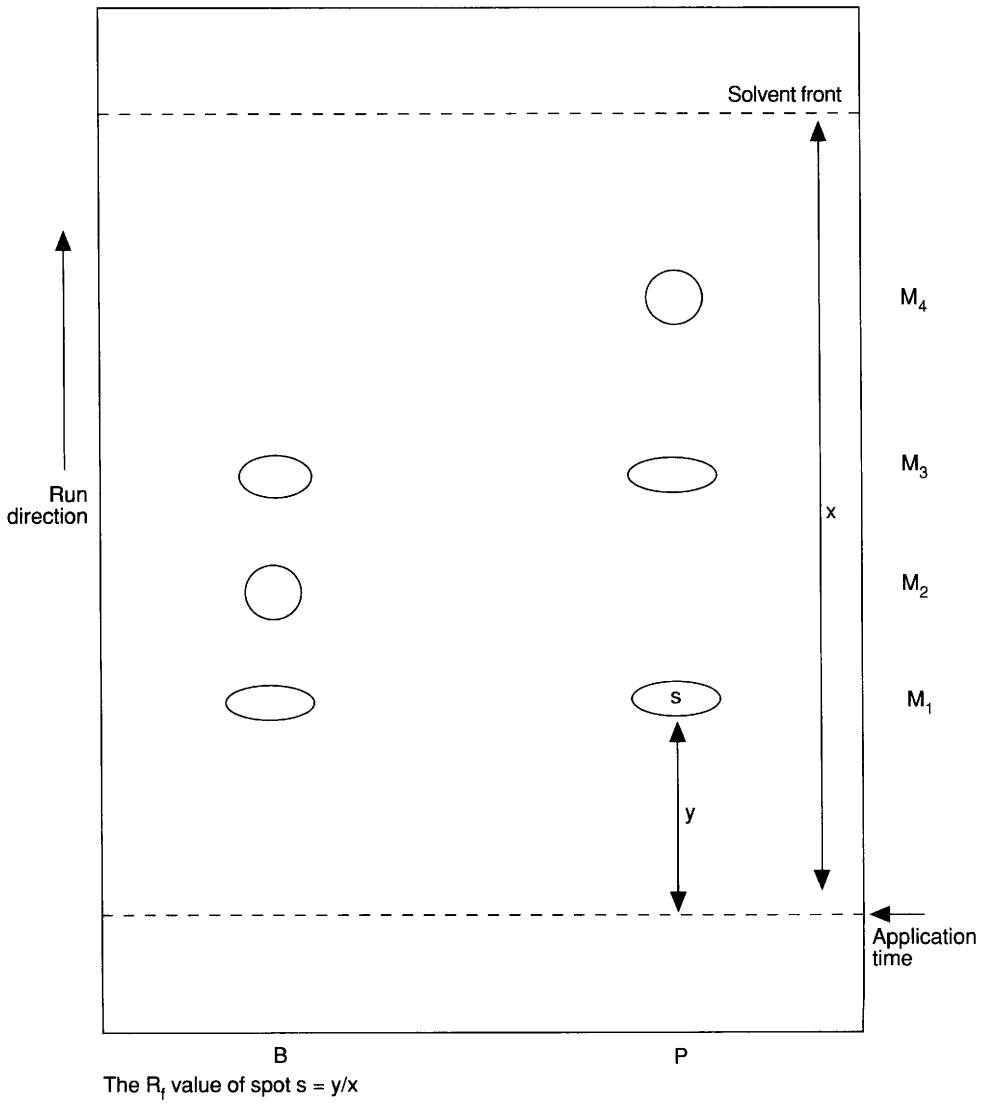

Figure 7 Autoradiogram of the metabolites of compound X

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This indicates that M_1 contains both rings of the original compound X since radioactivity is counted from both B (benzene ring labelled) and P (pyrimidine ring labelled). M_2 does not show up in the test with P (pyrimidine ring only labelled) but does in the test with B (benzene ring only labelled). This suggests that M_2 contains the benzene ring only. By similar reasoning we can deduce that M_3 has both rings and M_4, the pyrimidine ring only.

Note that metabolites M_2 and M_4 are both present after both tests (the tests were identical) and are separated in the chromatograms. They contain no radioactivity because they are from the unlabelled halves of both B and P; only metabolites with a radioactive label show up on the autoradiogram.

These results suggest that the ester linkage in compound X might have been hydrolysed to form two fragments (M_2 and M_4) each containing one of the rings. M_1 and M_3, in which the two rings remain joined, could be a derivative of compound X in which one of the ring positions has been hydroxylated or in which the $-CH_3$ group has been oxidised to $-CH_2OH$ or $-CO_2H$ (*Fig 8*).

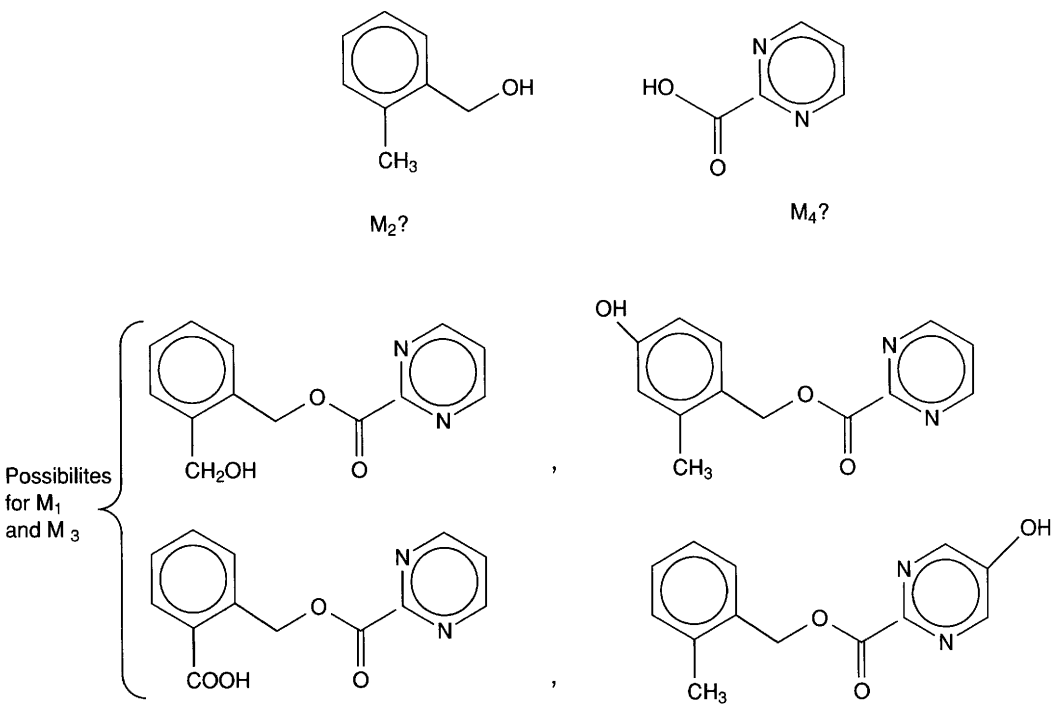

Figure 8 Possible metabolites of compound X

At this stage, of course, these are speculations which must be confirmed or rejected. For example, the proposed metabolites could be synthesised and TLCs run to find out if they give the same R_f values as M_1 to M_4. Alternatively, M_1 to M_4 could be isolated for identification by, for example, HPLC/MS (high performance liquid chromatography / mass spectrometry) in which each compound separated is fed directly into a mass spectrometer.

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The breakdown of azoxystrobin

Ultimately it should be possible to trace the breakdown of an agrochemical to carbon dioxide – the ultimate breakdown product of all carbon-containing compounds. For azoxystrobin, two breakdown mechanisms were found.

One was photolytic (*ie* light induced) and occurred on the surface of soil. The half life (*ie* the time for half the applied chemical to disappear) for azoxystrobin to break down to carbon dioxide by this process is about one to two weeks.

In the absence of light, azoxystrobin breaks down to carbon dioxide via microbial action with a half-life of about 80 days. This process results in the formation of an acid metabolite formed by hydrolysis of the ester group in azoxystrobin.

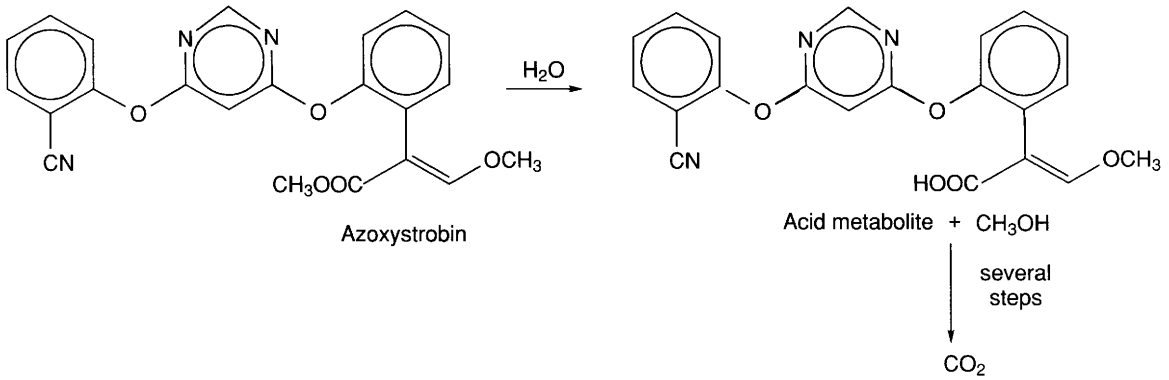

Figure 9 One step in the break down of azoxystrobin in the dark – hydrolysis of methyl ester to leave an acid

In the field, radiolabelling studies indicate that azoxystrobin breaks down with a half life of about 14 days and none of the acid metabolite is found. This suggests that photolysis is the main breakdown mechanism in the field. Similar results are found for the formulated product as for azoxystrobin alone. This rapid and complete breakdown is a very encouraging result as it indicates that there is little need to worry about long lived residues and breakdown products.

Mathematical modelling

Much of the data obtained from experimental work are used in mathematical models run on powerful computers which can help predict things such as the movement of agrochemical residues through ground water. This requires data about the chemical and its degradates (reactivity, solubility, volatility *etc*) as well as geological information about soil types and rainfall records over many years. The models themselves and the computers which run them are becoming increasingly sophisticated and it may soon become possible to predict residue levels in ground water in a particular place while a compound is still at the research stage. One Zeneca scientist has suggested that eventually such predictions could be made about hypothetical compounds which have not yet been made in the laboratory.

Registration

Before an agrochemical can be sold, it must fulfil the legal safety requirements of the country concerned, to ensure that it will not harm users, consumers of the crop or the environment. It must also be shown to be effective. Regulations vary from country to country and also depend on the circumstances of use. For example, a compound

could be registered for all crops or for just a range of crops. The registration agency requires the results of a vast number of experiments and will specify what experiments must be done, and how. There is normally a dialogue between the Zeneca scientists and the specialists of the regulatory agency to ensure that the product is acceptable. Registrations can be cancelled or modified after being granted if unforeseen problems arise. The cost of the whole process required to register a new agrochemical in Europe is *ca* £50 million (1996 prices) of which 17% goes on efficacy, 50% on human safety and 33% on the environment. Products already on the market are kept under continuous review as they must be re-registered periodically.

Azoxystrobin will go on sale in different countries at different times as registrations are obtained. Different names will be used depending on the country and the use for which it is being marketed. In Europe it is called Amistar when sold for use on cereal crops. This product was launched in early 1997 and is also sold as Heritage when used in the US for use on golf courses.

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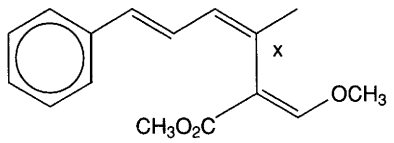
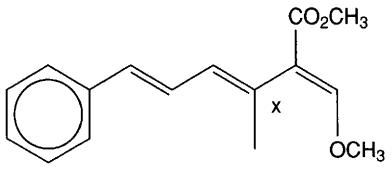
Answers to questions

1. See structures below. The difference is at the carbon atom marked x. In the correct structure, the methyl group and the chain containing the aromatic ring are *trans*- and in the incorrect structure, they are *cis*-. The two structures are isomers.



strobilurin A (correct structure)



incorrect structure

2. Nitrogen atoms can participate in hydrogen bonding with water molecules.

3. a) (i) Make labelled benzene as described in the box.
Convert this to methylbenzene by a Friedel-Crafts reaction with chloromethane and an aluminium chloride catalyst.

Oxidise the methyl group with acidified manganate(VII) ions to yield benzoic acid.

Esterify with methanol.

(ii) Make benzoic acid with the carboxyl carbon labelled as described in the box and then esterify with methanol.

(iii) Esterify benzoic acid (or benzoyl chloride) with labelled methanol obtained as described in the box.

b) Prepare labelled sodium cyanide as described in the box. React this with bromoethane to give propanenitrile with the label on the carbon of the –CN group. Acid hydrolysis will then yield propanoic acid labelled as required.

c) Make labelled benzene as described in the box. Nitrate with concentrated nitric and sulfuric acid to yield nitrobenzene which can be reduced to phenylamine with tin and hydrochloric acid for example.

Other synthetic routes are, of course, possible.

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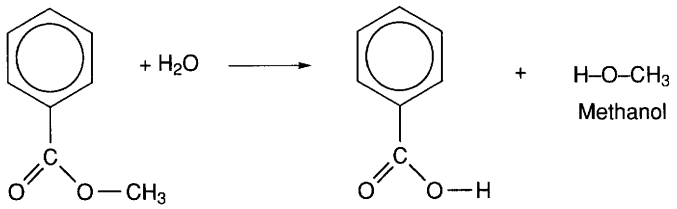
4.



Methyl benzoate Benzoic acid

Water is required and the reaction is catalysed by both acids and bases. In the latter case, the salt of benzoic acid is formed rather than the acid itself.