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Guanidinium and Amidinium Fungicides: A New Class of Carbocation Mimetic Ergosterol Biosynthesis Inhibitors

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Abstract: A novel class of chemical has been designed with the aim of inhibiting the Δ^{14}-reductase and Δ^{8}–Δ^{7}-isomerase enzymes in the ergosterol biosynthesis pathway in fungi. Use was made of knowledge about the mechanisms of both enzymes and the mode of action of known, fungicidal inhibitors of these enzymes. Pioneer examples have been synthesised and have been demonstrated to be potent inhibitors of ergosterol biosynthesis in *Ustilago maydis* (DC) Corda, acting in the same manner as the commercial fungicide fenpropimorph. They also showed excellent fungicidal activity against *Erysiphe graminis* DC f. sp. *hordei* Marchal (powdery mildew of barley) and *Puccinia recondita* Rob. ex Desm. (wheat leaf rust) in in-vivo glasshouse tests. Using these compounds as a starting point, systematic structural variation has been carried out. Testing of a wide range of analogues at high volume confirms the potential of this class of compound to control mildew and rust pathogens at levels comparable to those of the standards. Correlation of in-vivo and enzymatic data is good and the structure–activity relationship developed for this series of compounds closely parallels that found for the morpholine/piperidine class of fungicides, suggesting a common mode of action.

Key words: ergosterol biosynthesis, carbocation mimetic, fungicide, cereal pathogen.

1 INTRODUCTION

The success of interruption of the ergosterol biosynthesis pathway as a means of controlling pathogenic fungi in plants and mammals can easily be gauged by examination of the variety of fungicides and antimycotics available that exhibit this mode of action. In the control of temperate cereal diseases, for example, greater than 70%, by market share, of fungicides used are sterol biosynthesis inhibitors (SBIs). The most important class of fungicides in this group is those that inhibit C(14) demethylation. These include the triazole and imidazole fungicides such as propiconazole and prochloraz. A second class of chemistry that is also of major importance, especially in the control of powdery mildews on cereals (*Erysiphe* spp.), is the morpholine/piperidine class. This class of fungicide inhibits two enzymes in the biosynthesis pathway, Δ^{14}-reductase and, more importantly, Δ^{8}–Δ^{7}-isomerase.[1,2] The most widely used compounds of this type are the fungicides fenpropimorph and fenpropidin (Fig. 1). These compounds are highly active in both eradicant and protectant fashion against *Erysiphe graminis* DC and have

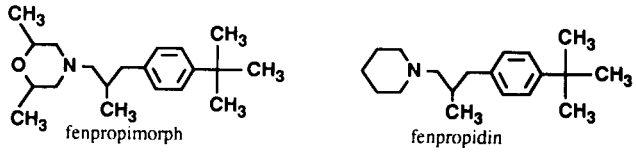

Fig. 1. Morpholine/piperidine fungicides of commercial importance.

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subsidiary activity against rusts (*Puccinia* spp.) and net blotch (*Pyrenophora teres* Drechs.).

Extensive work has been carried out on the structure–activity relationships of the morpholine fungicides against plant pathogens and in cell-free sterol synthesising preparations of fungi and higher plants.[3–8] For those compounds of this class described in the open literature, fenpropimorph and fenpropidin represent an optimum spectrum and level of activity. The most characteristic and important feature of these chemicals is the presence of a tertiary amine.

The mechanisms for the reduction of the Δ^{14} double bond (Fig. 2(a)) and, later in the pathway, the isomerisation of the Δ^{8} double bond (Fig. 2(b)) are similar, in that both mechanisms are thought to involve a carbocation as an intermediate state (bracketed).[3–5] There is considerable evidence to suggest that the morpholine/piperidine fungicides, protonated at physiological pH, act by mimicking, in both enzymes, the cationic high-energy intermediate.[1–5,7–9] They are in effect transition state analogues of the natural sterol substrate and consequently bind very strongly to the enzyme, making use of those enzyme interactions that help to stabilise the transition state and reduce the activation energy of the enzymatic reaction. This mode of inhibition was first postulated by Pauling.[10]

The concept of using an amine as a mimic for a carbocation has been exploited to design inhibitors of a variety of biochemical processes. Examples are Beneveniste's work on azadecalin inhibitors of squalene epoxide cyclase and cycloartenol-obtusifoliol isomerase, and Poulter's inhibitors of several of the stages on the isoprenoid pathway to squalene.[11–13] Work is currently

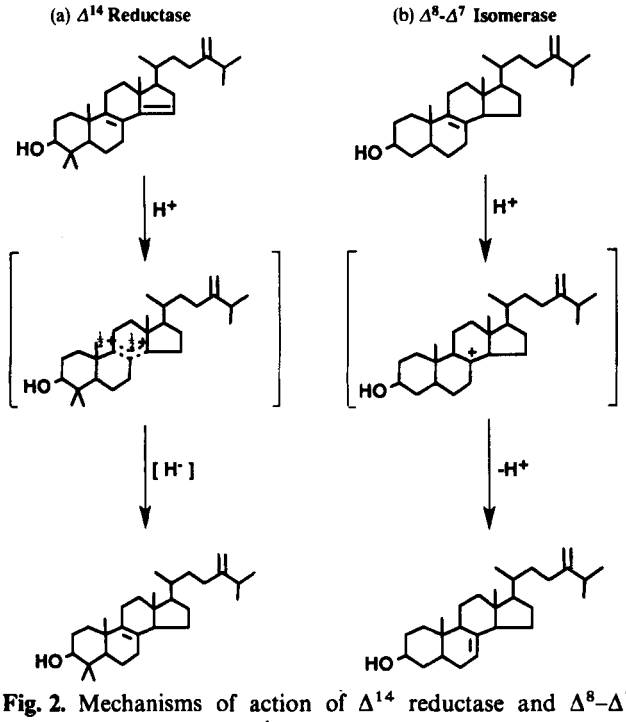


Fig. 2. Mechanisms of action of Δ^{14} reductase and Δ^{8}–Δ^{7} isomerase.

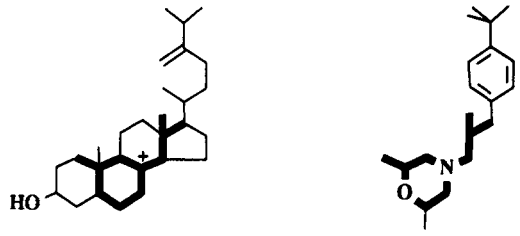


Fig. 3. Superposition of fenpropimorph over the isomerase carbocationic intermediate.

in progress to exploit this concept further in the fungicide area, by rationally designing other potent inhibitors to Δ^{8}–Δ^{7}-isomerase and Δ^{14}-reductase.[7,8] Some groups have gone a stage further and tried other methods for mimicking biochemically generated carbocations. For instance, sulfonium salts have been prepared that successfully inhibit various monoterpene synthases.[14]

Entry into this field was influenced by the intriguing question as to whether there existed an alternative method of inhibiting the Δ^{8}–Δ^{7}-isomerase and Δ^{14}-reductase enzymes. Of particular interest was the fact that a protonated amine is tetrahedral in shape whereas the carbocation transition state, for both enzymes, would normally be planar, and that this might suggest that an alternative planar carbocationic mimic might exhibit improved binding. The hope was that such a moiety could then lead to agricultural fungicides with improved activity or novel properties.

To aid this approach, use was made of a binding model for fenpropimorph that is illustrated in Fig. 3. This model, due to Baloch, was adopted for the successful manner in which it explains the structure–activity relationship of morpholine fungicides. Recently, several other groups have suggested and made use of a very similar model.[7–9,15]

2 DESIGN OF NOVEL CATIONIC MIMICS

Previous work had suggested the possibility of using amidinium species as mimics for oxonium intermediates in inhibiting glycosylase reactions.[16] This is another case where having a planar cation mimic was thought desirable. This prompted the examination of the possibility of using a cyclic guanidinium (or iminohexahydropyrimidinium) nucleus as the central structural feature in the new inhibitor type. This is exemplified in Fig. 4(a). Subsequently the cyclic amidinium (or iminopiperidinium) nucleus was also examined (Fig. 4(b)). Both these systems are guaranteed to be positively charged and planar at physiological pH. The

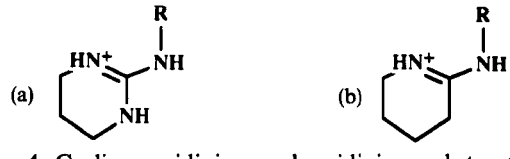


Fig. 4. Cyclic guanidinium and amidinium substructures.

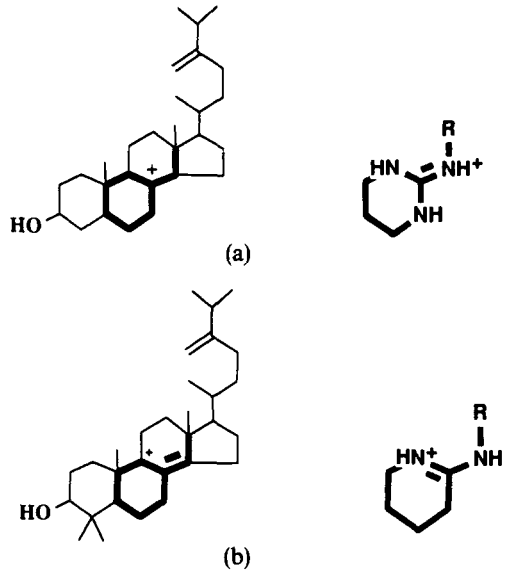


Fig. 5. (a) Superposition of cyclic guanidinium over the Δ^8–Δ^7 carbocationic intermediate. (b) Superposition of cyclic amidinium over the Δ^{14} reductase carbocationic intermediate.

binding concept was that the central carbon atom in the cationic system would take the place of the C(8) sterol carbon (Fig. 5(a)). Additionally the amidinium substructure was chosen with the specific aim, bearing in mind the model described in Fig. 3, of mimicking the delocalised allyl cation of the Δ^{14} reductase intermediate (Fig. 5(b)). Recently, a similar idea has been employed in the successful design of a squalene synthetase inhibitor.[17]

Initial synthesis was aimed at targets that included many of the known beneficial features of the morpholines/piperidines, whilst also incorporating both the guanidinium and amidinium substructures. Accordingly, lead compounds 1 and 2 were initially prepared (Fig. 6).

Subsequent to the synthesis and screening of compounds 1 and 2, structural variation around the lead compounds was carried out. Structural variation was

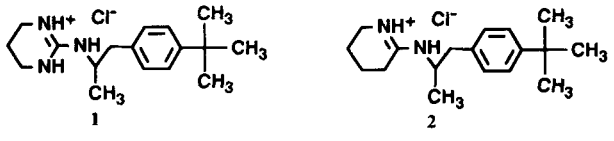


Fig. 6. Lead guanidinium and amidinium compounds.

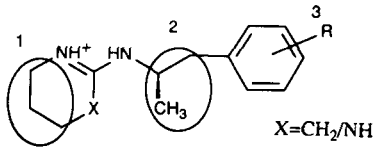
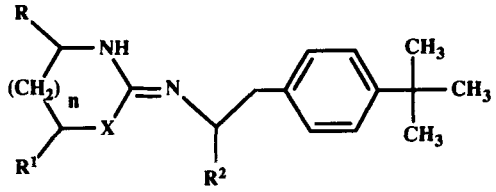


Fig. 7. Logical identification of loci for structural modification.



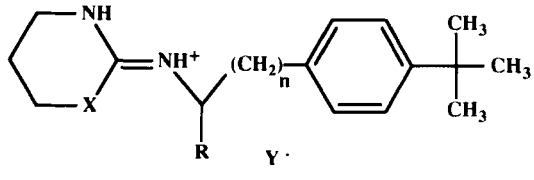
Compound	R	R^1	R^2	n	X	Form
3	H	H	CH_3	0	CH_2	free base
4	H	H	CH_3	0	CH_2	HCl salt
5	H	H	CH_3	1	CH_2	free base
2	H	H	CH_3	1	CH_2	HCl salt
6	CH_3	H	CH_3	1	CH_2	free base
7	CH_3	H	CH_3	1	CH_2	HCl salt
8	H	H	CH_3	2	CH_2	free base
9	H	H	CH_3	2	CH_2	HCl salt
1	H	H	CH_3	1	NH	HCl salt
10	CH_3 (cis)	CH_3	CH_3	1	NH	HCl salt
11	H	H	H	0	NH	HBr salt

Fig. 8. Series 1 compounds.

carried out in three areas (Fig. 7) and the compounds synthesised can be grouped into three separate series:

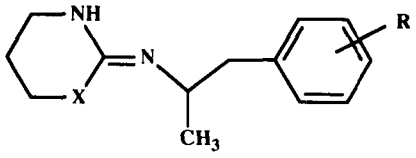
Series 1. Variation of size and substitution of the heterocyclic ring (Fig. 8).
Series 2. Variation of length and branching of the alkyl bridge (Fig. 9).
Series 3. Variation of substitution on the phenyl ring (Fig. 10).

Once synthesised, compounds were assayed in in-vitro tests designed to estimate their potency as inhibitors of the postsqualene sterol biosynthesis pathway in *Ustilago maydis* (DC) Corda. The compounds were also tested for their potency against *Erysiphe* and *Puccinia* species, in in-vivo glasshouse tests.



Compound	R	n	X	Y
12	H	0	NH	Cl
13	CH_3	0	NH	Cl
14	H	1	NH	Br
1	CH_3	1	NH	Cl
15	C_2H_5	1	NH	Cl
16	H	2	NH	Cl
17	CH_3	0	CH_2	Cl
18	H	1	CH_2	Cl
2	CH_3	1	CH_2	Cl

Fig. 9. Series 2 compounds.



Compound	X	R	Form
19	NH	4-C_2H_5	HCl salt
20	NH	4-$CH(CH_3)_2$	HCl salt
1	NH	4-$C(CH_3)_3$	HCl salt
21	NH	4-C_6H_{13}	HCl salt
22	NH	4-$Si(CH_3)_3$	HCl salt
23	NH	4-$Si(C_2H_5)(CH_3)_2$	HCl salt
24	NH	4-OC_4H_9	HCl salt
25	NH	4-C_6H_5	HCl salt
26	NH	3-OC_6H_5	HCl salt
27	NH	4-OC_6H_5	HCl salt
28	NH	4-$CH_2CH_2C_6H_5$	HCl salt
29	CH_2	4-C_2H_5	free base
30	CH_2	4-C_2H_5	HCl salt
31	CH_2	4-$CH(CH_3)_2$	HCl salt
5	CH_2	4-$C(CH_3)_3$	free base
2	CH_2	4-$C(CH_3)_3$	HCl salt
32	CH_2	4-C_6H_{13}	HCl salt
33	CH_2	4-$Si(CH_3)_3$	free base
34	CH_2	4-$Si(CH_3)_3$	HCl salt
35	CH_2	4-$Si(C_2H_5)(CH_3)_2$	free base
36	CH_2	4-$Si(C_2H_5)(CH_3)_2$	HCl salt
37	CH_2	4-OC_4H_9	HCl salt
38	CH_2	4-C_6H_5	free base
39	CH_2	3-OC_6H_5	free base
40	NH	3,4-$(CH)_4$	HCl salt
41	CH_2	3,4-$(CH)_4$	HCl salt

Fig. 10. Series 3 compounds.

3 EXPERIMENTAL

3.1 Synthesis

3.1.1 General

A three-step route to the initial target guanidines was selected (Fig. 11(A)). The normal intermediate substituted phenylpropylamine was prepared by standard amphetamine chemistry. Thus, a substituted benzaldehyde was condensed with a nitroalkane in the presence of ammonium acetate, and the resulting nitrostyrene reduced with lithium aluminium hydride.[18,19] Condensation of this amine, in the final step, with *S*-ethyl-*N*,*N*′-trimethylenethiuronium bromide (prepared from the cyclic thiourea) was not easy and only worked with α-unsubstituted amines. α-Substituted amines gave no desired product, only starting amine and amine ethylated on nitrogen. Clearly the amine was too sterically hindered to react well with the thiuronium salt.

To prepare α-substituted derivatives a four-step route was used (Fig. 11(B)). The amine was condensed with 2-chloropyrimidine and the resulting aminopyrimidine hydrogenated in the presence of 10% palladium on charcoal with an equivalent of acid (normally hydrochloric) added.[20,21] This route allowed a certain flexibility in the choice of substitution on the tetrahydropyrimidine ring. The free guanidine could be obtained from the salts thus produced by treatment with concentrated sodium hydroxide. All guanidines were, however, tested in the salt form.

The amidines were, in general, prepared by condensation of the same amine intermediates as used in the

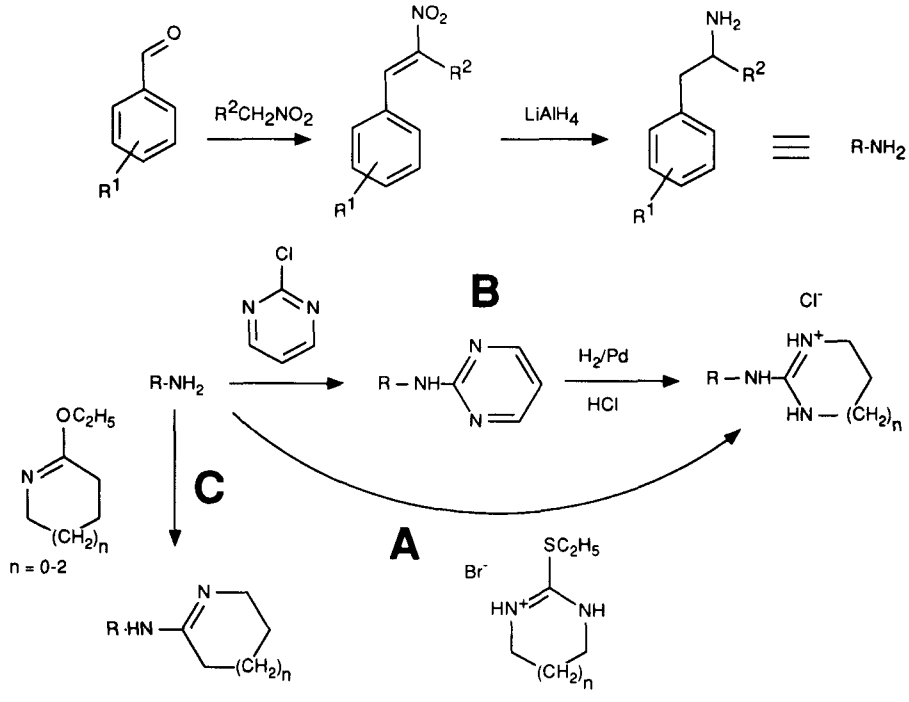


Fig. 11. General synthetic routes.

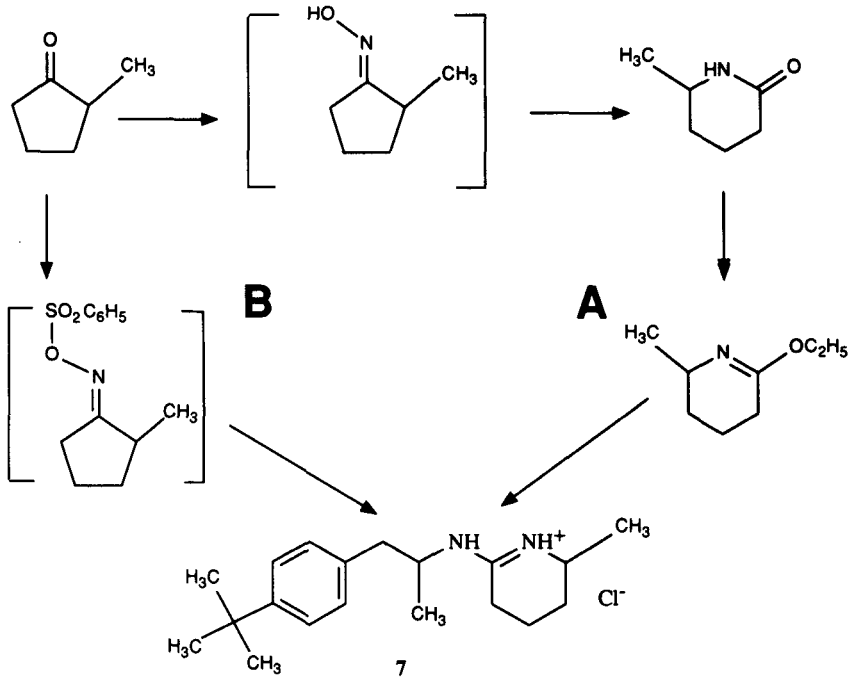


Fig. 12. Synthetic route to 2-(1-(4-*tert*-butylphenyl)prop-2-ylimino)-6-methylpiperidine hydrochloride, **7**.

guanidine synthesis with a cyclic imidate (Fig. 11(C)).[22] This method of preparation was employed when the attempted condensation of a lactam and a primary amine in the presence of a chlorophosphorous reagent, a more standard route to amidines, was found to be unsuccessful. The cyclic imidates could be prepared by treatment of the appropriate lactam with ethyl chloroformate.[23]

The amidines could be prepared either as the hydrochloride salts or converted to the free bases. The NMR spectra of the free bases were easy to interpret; those of the salts were considerably more complex and implied that a two-component mixture was generally present. This can be taken to indicate that, for the salt, there is no free rotation on the NMR time scale around the exocyclic C–N bond and, therefore, *cis/trans* isomerism exists. In the free base, free rotation is possible.

Where synthetic techniques other than those described above were used, they are described in the following sections. Representative preparative details are presented in the experimental section. Full spectral and characterisation data are also documented.

3.1.2 Series 1

The appropriate lactam starting for the α-methyl amidine salt 7 was not available. This material could be prepared starting with α-methylcyclopentanone, going through a Beckmann rearrangement and then using the standard procedure (Fig. 12(A)). However, the overall yield for this sequence was poor. It was found that the one-pot condensation/rearrangement, due to Oxley and Short, from the same starting material could be used (Fig. 12(B)).[24] This gave acceptable yields (30%) of the amidine salt.

3.1.3 Series 2

The general routes described above could be used for all Series 2 compounds. The synthesis of some compounds required the preparation of several amine precursors by standard methods. The unknown 4-*tert*-butylphenylpropylamine, 42, was prepared from 4-*tert*-butylcinnamonitrile by reduction. Attempts to effect a one-step reduction were unsuccessful. Thus, reacting the nitrile with lithium aluminium hydride gave only polymer. However, hydrogenation over palladium followed by reduction with borane in tetrahydrofuran gave the desired material (Fig. 13).

3.1.4 Series 3

The general preparative route outlined earlier could be used to synthesise all Series 3 guanidines and amidines. The substituted phenethylamines required could all be

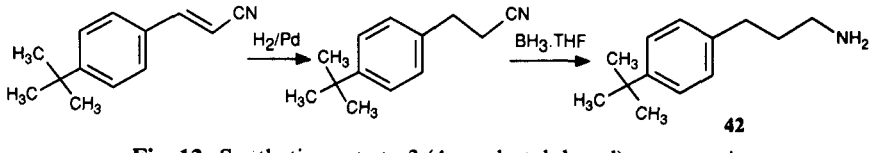


Fig. 13. Synthetic route to 3-(4-*tert*-butylphenyl)propanamine.

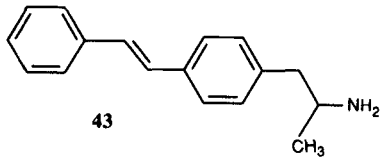


Fig. 14. 1-(4-(2-phenylethyl)phenyl)propan-2-amine.

synthesised by the standard route. This route worked well in nearly all cases.

The 4-(phenylethyl)phenyl guanidine, 28, was prepared using the stilbylpropylamine 43 (Fig. 14). In the final hydrogenation step the stilbyl double bond was reduced along with the pyrimidine.

The starting alkylsilylbenzaldehydes 44 and 45, for the preparation of 22, 23 and 33–36 were not available commercially. These very easily air-oxidised materials were, therefore, prepared from 4-bromobenzaldehyde using a protection/deprotection sequence (Fig. 15).

3.2 Experimental methods

3.2.1 Chemistry

Melting points were recorded on an Electrothermal melting point apparatus and are uncorrected. Infra-red spectra were recorded on a Perkin–Elmer 1420 Ratio Recording infra-red spectrophotometer. [^{1}H]NMR and [^{13}C]NMR were run either at 90 MHz on a Varian EM 390, at 250 MHz on a Bruker AC-250 or, occasionally, at 500 MHz on a Bruker AM-500 spectrometer. Microanalyses were carried out by Butterworths Laboratories Ltd, Middlesex, UK. Solvents were generally purchased dry, from Aldrich Chemical Company, or dried before use. All chemicals were purchased either from Aldrich Chemical Company or Lancaster Synthesis and, unless otherwise stated, were used without further purification.

3.2.1.1 General preparation of aminopyrimidines. Equimolar quantities of the amine, 2-chloropyrimidine and diisopropylethylamine were dissolved in dry pentanol and heated at reflux 4–8 h, the reaction being monitored by reverse phase HPLC (acetonitrile + water, 85 + 15 by volume). The solvent was then removed under reduced pressure, the residue taken up in chloroform or dichloromethane, washed with 2 M sodium hydroxide and water, dried, filtered and the solvent removed under reduced pressure. If the residue was not crystalline it was generally purified by column chromatography (silica, ethyl acetate + hexane).

N-(1-(4-*tert*-butylphenyl)prop-2-yl-2-aminopyrimidine, yield, 53%, was a colourless solid recrystallised from hexane, m.p. 68·0°C. Found C, 75·9; H, 8·80; N, 14·5%; required C, 75·8; H, 8·60; N, 15·6%, ν_{max}(neat) 3266, 2965, 1591, 1535, 1452 cm^{-1}, δ_H (deuterchloroform) 8·13 (2H, d, J = 5 Hz), 7·26 (2H, d, J = 8 Hz), 7·13 (2H, d, J = 8 Hz), 6·38 (1H, t, J = 5 Hz), 4·39 (1H, hept, J = 7 Hz), 2·83 (2H, m), 1·3 (9H, s), 1·16 (3H, d, J = 7 Hz).

3.2.1.2 General preparation of 2-iminohexahydropyrimidine hydrochlorides. In general, the aminopyrimidine was dissolved in a 2 + 1 or 3 + 1 (by volume) mixture of ethanol and hydrochloric acid. Palladium, 10% charcoal, 5–10% by weight, with respect to substrate, was added and the mixture hydrogenated at atmospheric pressure and temperature until hydrogen uptake had ceased, generally after two molar equivalents of hydrogen had been absorbed. The mixture was passed through a plug of celite and the solution concentrated under reduced pressure and then diluted with water. The mixture was then washed with ether and extracted twice with chloroform. The organic extracts were washed (with water), dried, filtered and the solvent removed under reduced pressure to afford crude product.

N-(1-(4-*tert*-butylphenyl)prop-2-yl)-*N'*,*N''*-trimethyleneguanidinium hydrochloride, (**1**), yield, 55% of a powder, m.p. 169·1°C after recrystallisation from methyl ethyl ketone. Found C, 65·1; H, 8·60; N, 13·6%; required C, 65·9; H, 9·10; N, 13.6%, ν_{max} (potassium bromide) 3286, 3212, 3159, 3052, 2972, 1645 cm^{-1}, δ_H (deuterochloroform) 7·9 (2H, brs, diss. on deuterium oxide shake), 7·53 (1H, d, J = 8 Hz, diss. on deuterium oxide shake), 7·34 (4H, s), 4·25 (1H, hept, J = 7 Hz), 3·20 (4H, m), 2·8 (2H, m), 1·72 (2H, quin, J = 6 Hz), 1·27 (9H, s), 1·20 (3H, d).

3.2.1.3 General preparation of substituted phenylpropyliminopiperidine hydrochlorides. In general the appropriate amine was heated with 2-ethoxy-3,4,5,6-tetrahydropyridine (1.2–1.5 equiv.) in the absence of solvent, under nitrogen at 140°C. A reaction time of 7 h was used. The cooled mixture was taken up in 4M hydrochloric acid and washed twice with ether, the

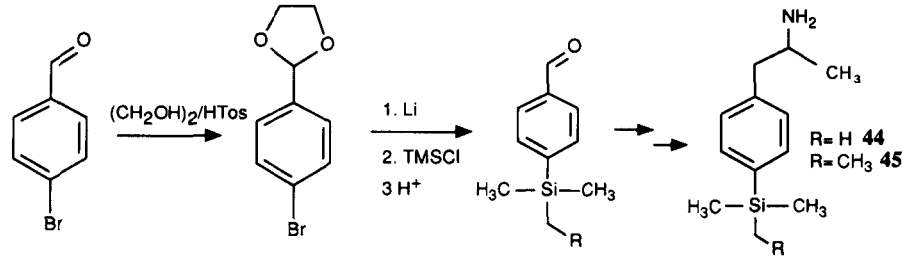


Fig. 15. Synthetic route to 1-(4-(trialkylsilyl)phenyl)propan-2-amine.

washings being discarded, and then extracted twice with chloroform. The chloroform layers were combined, washed twice with water and the washings back-extracted. The organic part was dried using sodium sulfate. The mixture was filtered and the solvent removed under reduced pressure. The residue was then triturated with methyl ethyl ketone, affording, in certain cases, a crystalline hydrochloride salt.

The amidine free base could be generated by treating a cold aqueous solution of the salt with 5M sodium hydroxide, extracting the mixture with either, washing the organic layer once with water, drying over sodium sulfate, filtering and removing the solvent under reduced pressure.

N-(1-(4-*tert*-butylphenyl)prop-2-yl)-2-iminopiperidine hydrochloride (**2**), yield 60%, m.p. 190·3°C after recrystallisation from methyl ethyl ketone. Found C, 69·70; H, 9·30; N, 9·15%, required C, 70·0; H, 9·45; N, 9·1%, ν_{max} (potassium bromide) 3400, 3212, 3052, 2965, 1658 cm^{-1}.

3.3 In-vivo biological screening

Compounds were tested *iv vivo* against *Erysiphe graminis* DC sp. *hordei* Marchal (barley powdery mildew) and *Puccinia recondita* Rob. ex. Desm. (wheat leaf rust). All secondary tests against these pathogens were carried out with fenpropimorph as a standard.

3.3.1 Test for protectant and eradicant activity against E. graminis *f. sp.* hordei

Inoculum was produced by infecting stock plants, sown 8 per 7·5-cm pot in peat-based compost, of barley (*Hordeum vulgare* L., cv. Golden Promise). Test plants of the same cultivar produced in the same manner were used at 7–10 days old. Test and stock plants were inoculated by dusting conidia onto the plants and incubating at 18–20°C, RH 60–70% for 7–10 days.

Plants were sprayed with a test solution of chemical in acetone + water (10 + 90 by volume). Plants were sprayed 24 h before inoculation for a protectant test and 48 h after inoculation for an eradicant test. The test concentrations used were 400, 100, 50, 25 and 10 mg $litre^{-1}$.

Seven days after spraying the percentage leaf area infected was scored and the control for each compound calculated as a percentage of the infection of acetone/water control plants.

3.3.2 Test for protectant activity against P. recondita

Seed trays of thickly sown wheat (*Triticum aestivum* L., cv. Tonic) at the one-to-two-leaf stage were inoculated with a suspension of urediospores ($0·5 \times 10^6$ ml^{-1}) in water containing 1 ml $litre^{-1}$ 'Tween' 20. After inoculation, the trays were incubated at 100% RH, 20°C for 48 h prior to incubation in the glasshouse at 60–70% RH, 20°C for a further seven days. Spores were then collected with a vacuum spore collector. For screening, wheat cv. Tonic (sown 8 per 7·5-cm pot) was inoculated at 7–10 days old. A suspension of urediospores (prepared as above) was applied to test plants to give a complete cover of discrete droplets on all leaves.

Plants were sprayed with a test solution of chemical in acetone + water (10 + 90 by volume). Plants were sprayed 24 h before inoculation. The test concentrations used were 400, 100, 50, 25 and 10 mg $litre^{-1}$.

Seven days after spraying, the percentage leaf area infected was scored and the control for each compound calculated as a percentage of the infection of acetone/water control plants.

3.4 Ergosterol biosynthesis inhibition

Selected compounds were screened for inhibitory activity on the postsqualene ergosterol biosynthesis pathway in two separate assays designed to monitor the incorporation of radiolabelled acetate or mevalonate into membrane sterols of the smut, *Ustilago maydis* (DC) Corda. In one of these assays a cell-free preparation was used, as described by Peacock and Goosey.[25] For the second assay a whole-cell preparation was employed.

IC_{50} values for postsqualene inhibition of ergosterol biosynthesis were obtained, for both assays, by testing a compound at a range of concentrations (generally from 0·01 μM to 100 μM) and plotting the integrated HPLC peak area due to radioactivity incorporation into ergosterol against compound concentration and estimating the concentration at which incorporation was reduced to 50% of that of the control. Compounds were, in general, assayed at least twice and the average IC_{50} values calculated. Good reproducibility was obtained, the estimated standard error on these values being 20%.

Compounds were initially tested at 100 μM and were only tested at lower concentrations if they demonstrated significant activity. In several cases it was found that total incorporation into sterols was much reduced, but that the sterol profile was unchanged from that of the control. The cause of this was not established. Since these compounds clearly had no effect on the sterol biosynthesis pathway, postsqualene, they were rated inactive.

3.4.1 Cell-free enzymic assay

The methods used were those described by Peacock and Goosey.[25] Liquid cultures (1 litre) of *U. maydis* in 2·8-litre Erlenmeyer flasks were grown in YED media (1·5% D-glucose, 0·3% yeast extract) and shaken in a rotary incubator at 120 rev min^{-1} at 24°C for 15–18 h. The cells (sporidia) were harvested by centrifugation at 1390*g* for 20 min and washed three times with equal volumes of homogenisation medium at 4°C.

The washed cells were suspended in 25 ml of homogenisation medium (2-[4-(2-hydroxyethyl)piperazin-1-yl] ethane sulfonic acid (HEPES), 50 mM, pH 7·4; ethylenediaminetetra(acetic acid) (EDTA), 1 mM; dithiothreitol, 5 mM; soybean trypsin inhibitor, 100 mg litre^{-1} and phenylmethyl sulphonyl fluoride, 100 mg litre^{-1}) and an equal volume of glass beads. The cells were disrupted by two 45-s periods of homogenisation at 4°C in a bead beater (Biospec Products). The resultant homogenate was centrifuged at 10000*g* for 15 min and the supernatant (S_{10}) used for enzyme assays.

The standard reaction mixture for measuring incorporation of DL-[2-^{14}C]mevalonic acid into non-saponifiable lipids was: 0·5 ml tissue (S_{10}) (8–12 mg protein ml^{-1}), DL-[2-^{14}C]mevalonic acid, 3 mM (0·2 μCi assay^{-1}); nicotine-adenine dinucleotide (NAD), 1·7 mM; adenosine 5'triphosphate (ATP), 3·5 mM; nicotine-adenine dinucleotide phosphate (NADPH), 1·7 mM; *S*-adenosyl-L-methionine, 1·7 mM; flavin-adenine dinucleotide (FAD), 0·17 mM; magnesium chloride, 3·5 mM; manganese (II) chloride, 3·5 mM in a total volume of 0·575 ml. The assay vials were incubated with shaking at 30°C for 3 h. The reaction was terminated by the addition of potassium hydroxide in ethanol (150 g litre^{-1}; 0·5 ml), the mixture was heated at 70°C for 30 min and then extracted with two 1-ml aliquots of hexane. The dried samples were redissolved in 150 μl of hexane and analysed by normal phase HPLC using a 25-cm, 3-μm silica column with a mobile phase of 0·022% (v/v) methanol in dichloromethane flowing at 1 ml min^{-1}. Radioactive sterols were detected using an on-line radiochemical HPLC detector (Berthold LB 506C).

DL-[2-^{14}C]mevalonic acid DBED (*N*,*N*-dibenzyl-ethylenediamine salt) (48·6 mCi mmol^{-1}) was obtained from DuPont (UK), New Research Products, Stevenage, UK. All analytical and chemical reagents were obtained from Sigma, Poole, UK.

3.4.2 Whole-cell enzymic assay

Liquid cultures (1 litre) of *U. maydis* in 2·8-litre Erlenmeyer flasks were grown in YED media (1·5% D-glucose, 0·3% yeast extract) and shaken in a rotary incubator at 120 rev min^{-1} at 24°C for 15–18 h. Ten microlitres of this culture were added to 1 ml of fresh medium in sterile multiwell plates. Inhibitors, dissolved in 10 μl of ethanol, were added as appropriate, and finally 1 μCi (86 nmoles) of [2-^{14}C]sodium acetate in ethanol were added to each mini-culture. The plates were incubated overnight at 24°C with shaking.

A portion (0·8 ml) of each culture was subsequently transferred to a test-tube with potassium hydroxide in methanol (100 g litre^{-1}; 0·8 ml) and heated to 60°C for 45 min to saponify lipids. Non-saponifiable lipids were extracted twice into hexane (1·5 ml) and evaporated to dryness under nitrogen.

The dried samples were redissolved in 150 μl of hexane and analysed by normal phase HPLC using a 25-cm, 3-μm silica column with a mobile phase of 0·022% methanol in dichloromethane flowing at 1 ml min^{-1}. Radioactive sterols were detected using an on-line radiochemical HPLC detector (Berthold LB 506C).[25]

The [2-^{14}C]sodium acetate (specific activity 55 mCi mmol^{-1}) was purchased from NEN Research Products, Dupont. All analytical and chemical reagents, including HPLC grade solvents, were purchased from FSA.

4 RESULTS AND DISCUSSION

4.1 Biological tests

It was not possible to screen compounds from any one series in a comparative fashion. Therefore, in order to compare activities and allow for variation between tests, each compound has been qualitatively ranked alongside the performance of the standard in the same test. These comparisons are tabulated in Tables 1–3. Results of a single comparative eradicant test of compounds 1 and 2 alongside fenpropimorph are displayed in Fig. 16.

4.2 Biochemical assays

The biochemical assays can be used in two ways. First, a sterol profile can be matched to the effect of a test compound. This can be compared with a control profile and profiles given by known SBIs, to establish whether the compound is an inhibitor and, also, to identify at which enzyme sites inhibition may be occurring.[25] The site of inhibition is normally identifiable by observation of increased radiolabel incorporation into an immediate precursor. Secondly, the quantity of labelled ergosterol

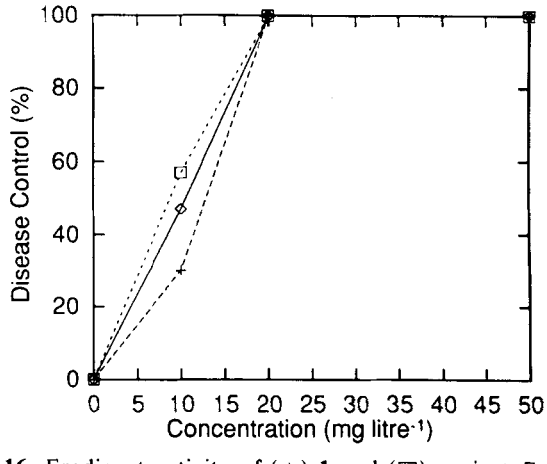


Fig. 16. Eradicant activity of (+) **1** and (□) against *E. graminis* f. sp. *hordei*: screened alongside (◇) fenpropimorph.

TABLE 1
Comparison of In-Vivo and Biochemical Activity of Series 1 Compounds

	Activity[a] against			
	E. graminis *f. sp.* hordei			IC_{50} (μM)
	Prot.	*Erad.*	P. recondita	U. maydis
4	+ +	+ + +	−	110
5	+ + + +	+ + + +	+ +	12
2	+ + + +	+ + + +	+ + +	20
6	+ + +	+ + +	+ + +	20
7	+ + + +	+ + +	−	3·5
8	+ + +	+ + +	−	60
1	+ + + +	+ + + +	+ + +	30
10	+ + +	+ + +	−	2
11	+	−	+ + +	100
Fenpropimorph	+ + + +	+ + + +	+ +	0·35

[a] + + + + LD_{90} <10 mg litre^{-1}. + + + LD_{90} 50–10 mg litre^{-1}. + + LD_{90} 100–50 mg litre^{-1}. + LD_{90} 400–100 mg litre^{-1}. − LD_{90} >400 mg litre^{-1}.

can be assayed, against the control, at a range of inhibitor concentrations in order to obtain a dose response curve and an IC_{50} representing the concentration at which incorporation into ergosterol is reduced to 50% of that of the control. This figure is not necessarily representative for any single enzyme in the pathway. It does, however, give a good measure of the overall sterol biosynthesis inhibiting capability of the test compound.

Representative HPLC traces (Fig. 17), compare the effect of fenpropimorph at 10 μM and 2 at 50 μM, on the sterol profile in *U. maydis*, against that when no compound was added. The major peaks observed in the control are identified in accordance with previous work.[25]

IC_{50} values for inhibition of ergosterol biosynthesis by **1** and **2** and by fenpropimorph, measured in both cell-free and whole-cell assays, are shown in Table 4.

IC_{50} values for inhibition of ergosterol biosynthesis, as measured in the whole-cell assay, have, where available, been tabulated alongside the efficacy data in Tables 1–3.

4.3 General discussion

When tested in an in-vivo test, sprayed to run-off, against *E. graminis*, it was found that compounds **1** and **2** were both fungicidally active and performed at an efficacy that was very comparable to that of fenpropi-

TABLE 2
Comparison of In-Vivo and Biochemical Activity of Series 2 Compounds

	Activity[a] against			
	E. graminis *f. sp.* hordei			IC_{50} (μM)
	Prot.	*Erad.*	P. recondita	U. maydis[b]
12	+ + +	+ +	−	In
13	+	+	+ +	In
14	−	+	−	In
1	+ + + +	+ + + +	+ + +	30
15	+ +	+ +	−	NT
16	+ + +	+ + +	+ + +	>100
17	+	+	+ + +	In
2	+ + + +	+ + + +	+ + +	20
Fenpropimorph	+ + + +	+ + + +	+ +	0·35

[a] + + + + LD_{90} <10 mg litre^{-1}. + + + LD_{90} 50–10 mg litre^{-1}. + + LD_{90} 100–50 mg litre^{-1}. + LD_{90} 400–100 mg litre^{-1}. − LD_{90} >400 mg litre^{-1}.
[b] In = Inactive at 100 μM. NT = Not tested.

TABLE 3
Comparison of In-Vivo and Biochemical Activity of Series 3 Compounds

	Activity[a] *against*			
	E. graminis *f. sp.* hordei			
	Prot.	*Erad.*	P. recondita	IC_{50} (μM) U. maydis[b]
19	+	+ + + +	+	NT
20	+ +	+ +	−	>100
1	+ + +	+ + + +	+ + +	30
21	−	−	−	In[c]
22	+ +	+ + +	+ + +	20
24	+ + +	+ + + +	+	In
25	+	+ +	+ + +	In
26	−	−	+ +	In
27	+ +	+ +	−	In
28	−	−	+ + +	In[c]
29	−	−	−	In
30	+	+ +	−	NT
31	+ +	+ + + +	−	>100
5	+ + + +	+ + + +	+ + +	12
2	+ + +	+ + + +	+ + +	20
32	−	−	−	NT
33	+ + +	+ + +	+ + + +	20
34	+ + +	+ + +	+ + +	NT
35	+ + + +	+ + + +	+ + + +	35
36	+ + + +	+ + + +	+ + + +	NT
37	+	+ + + +	−	In[d]
38	+ +	+ +	−	In
39	−	−	−	In
40	−	+ + + +	+ +	In
41	−	−	+	In
Fenpropimorph	+ + + +	+ + + +	+ +	0·35

[a] + + + + LD_{90} <10 mg litre^{-1}. + + + LD_{90} 50–10 mg litre^{-1}. + + LD_{90} 100–50 mg litre^{-1}. + LD_{90} 400–100 mg litre^{-1}. − LD_{90} >400 mg litre^{-1}.
[b] In = Inactive at 100 μM. NT = Not tested.
[c] 90% redn in total sterol incorp.
[d] 75% redn in total sterol incorp.

morph (Fig. 16). This was the case for both protectant and eradicant application.

When assayed in the cell-free sterol biosynthesis assay, both compounds **1** and **2** successfully inhibited ergosterol production at sub-micromolar levels (Table 4). This level of inhibition was found to be very similar to that of fenpropimorph in the same assay and is consistent with these compounds being analogues of high-energy enzyme intermediates. The amidinium salt, **2**, showed slightly higher activity than the guanidinium salt.

TABLE 4
Activity of Compounds **1** and **2** and Fenpropimorph against Sterol Biosynthesis in *U. maydis*

	IC_{50} (μM)	
	Cell-free assay	*Whole-cell assay*
1	0·73	30
2	0·13	20
Fenpropimorph	0·22	0·35

When assayed in the whole-cell assay, **1** and **2** again inhibited ergosterol biosynthesis, although now the level of activity appeared to be diminished by approximately two orders of magnitude. Fenpropimorph maintained a similar level of activity in both cell-free and whole-cell assays. Further analysis of the mode of action of **1** and **2** in the whole-cell assay suggests that at low to moderate concentrations, they inhibit only one enzyme in the pathway. This was, in general, found true for almost all analogues tested. Characteristically, an increase in the level of incorporation of label into a single peak, due to an abnormal sterol, was observed (Fig. 17(b) and (c)). The retention time of this peak was consistent between

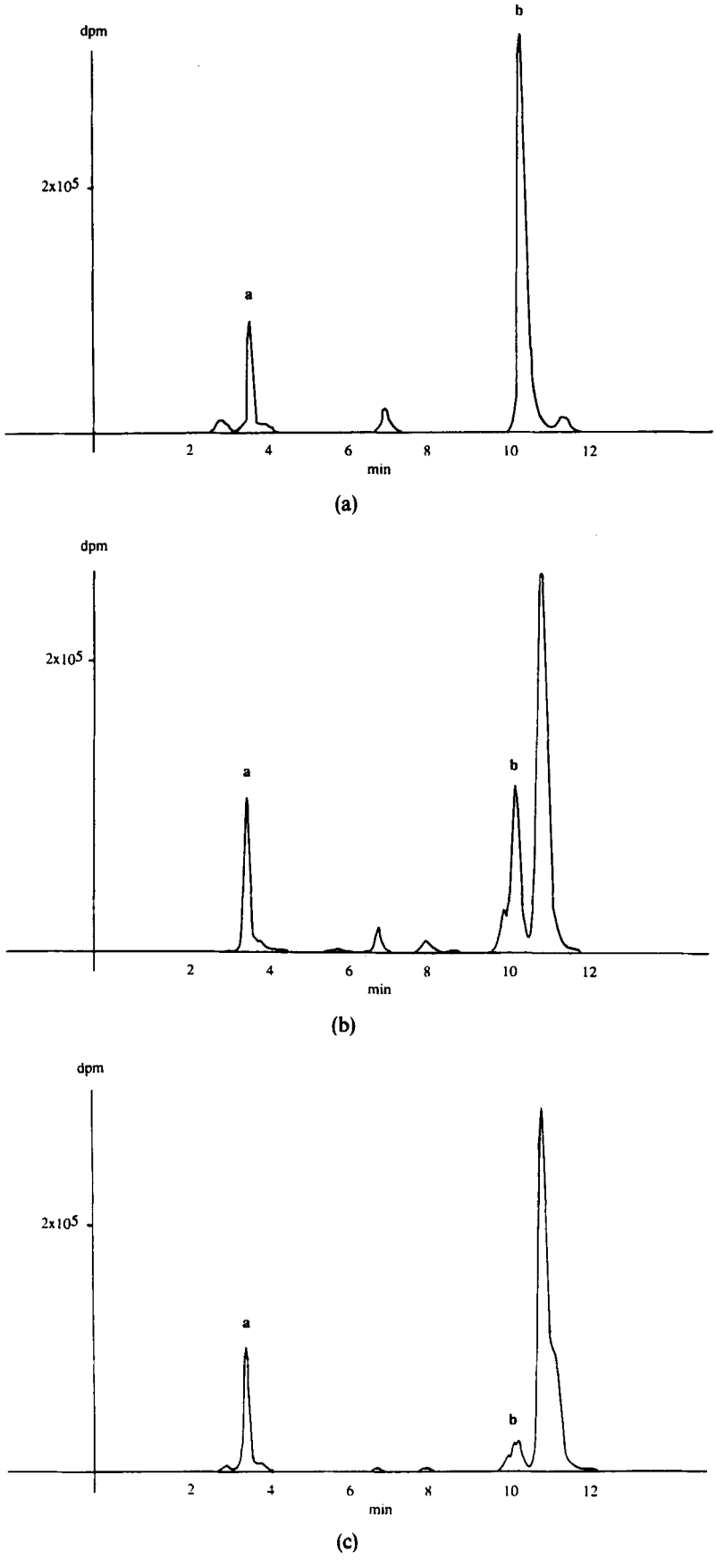


Fig. 17. HPLC trace illustrating radioactivity incorporation into sterols of *U. maydis*: (a) control, peak a is squalene, peak b ergosterol, (b) in the presence of 50 μM of compound **2**, (c) in the presence of 10 μM fenpropimorph.

analogues. Appearance of the abnormal sterol was concomitant with reduction of incorporation into ergosterol. The dose response curves calculated for compound **2** and the highly active 2-methyl analogue of **2**, compound **7**, illustrate this (Fig. 18).

Fenpropimorph at 10 μM exhibited a very similar sterol profile to **1** and **2**, suggesting that **1**, **2** and fenpropimorph have a common mode of action (Fig. 17(c)). On the basis of these profiles alone it is not possible to determine whether the mode of action is at the Δ^8–Δ^7-isomerase or Δ^{14}-reductase steps, as fenpropimorph is capable of inhibiting both enzymes.

The difference in IC_{50} values for **1** and **2** in the cell-free and whole-cell assays can be explained. The IC_{50} values, calculated in the whole-cell assay, do not necessarily reflect actual intracellular inhibitor concentrations, and it may be that there is a barrier to penetration or some other factor that reduces the effective intracellular concentration in the case of the polar and highly basic guanidine/amidine compounds (pK_a of 10–12) and renders them less active in the whole-cell assay. Fenpropimorph has a much lower pK_a of 7, despite being a tertiary amine, and is therefore able to cross membranes, in vivo, in un-ionised form.[26]

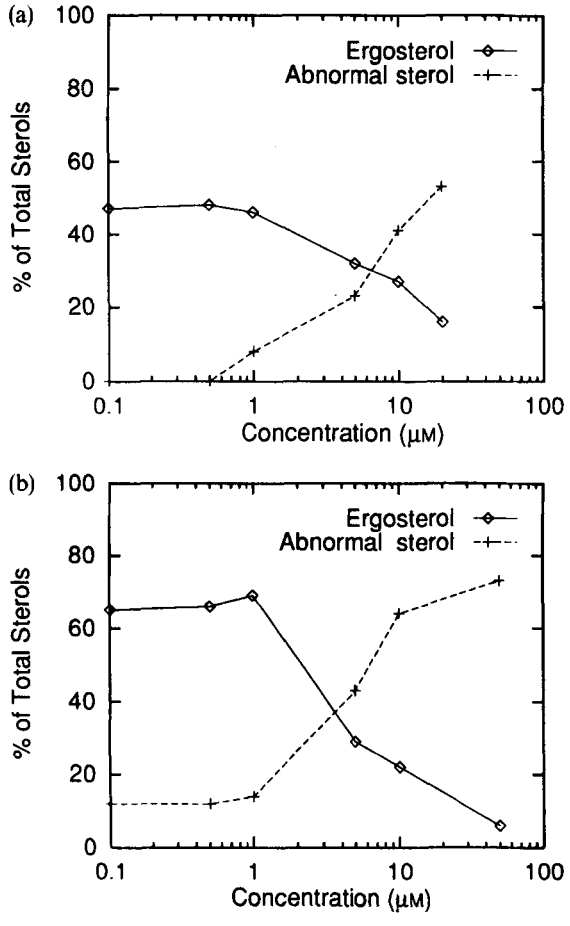


Fig. 18. Concentration dependence of incorporation of radioactivity into ergosterol on incubation of *U. maydis* with (a) compound **2**, (b) compound **7**.

It is not clear why these compounds should perform well *in vivo*, despite their poor apparent penetration into *U. maydis* cells. It may be a function of organism, with the implication that intracellular uptake of these compounds by *E. graminis* may be easier than it is by *U. maydis*.

The demonstration of good bioactivity of compounds 1 and 2, and the similarity of their behaviour to that of fenpropimorph, successfully justified the original design concept.

Activity in the morpholine/piperidine class is very dependent on the stereochemistry and nature of substitution on the heterocyclic ring and the propylene chain, and this suggests that many of the preferred features for activity optimise the shape and conformation of the molecule so as to make it most suitable for enzyme binding.[6,7] Features that change the physical properties of the molecule to aid factors such as transport and absorption are less significant. An important point to establish, therefore, was whether the activity of the guanidinium/amidinium class followed a similar pattern.

4.4 Series 1—Variation in ring size and substitution

4.4.1 Activity v. E. graminis

Against *E. graminis*, almost all compounds performed very well both in protectant and eradicant fashion to such an extent that it is difficult to separate them in terms of activity (Table 1). In general the activity exhibited was similar to that of **1** and **2** in terms of spectrum and efficacy. Reducing the ring size from six reduced activity; for example **4** was markedly less active than **2**, and **11** was almost totally inactive. Increasing the ring size to seven, as in **8**, did not reduce activity as much. Introduction of methyl substitution at the 2 (and 4) position maintained excellent mildew activity (**7**, **10**) and perhaps it should be pointed out that the *cis*-dimethyl guanidine **10** is the direct guanidine analogue of fenpropimorph.

4.4.2 Activity v. P. recondita

Against *P. recondita* more variability in activity was observed (Table 1). The compounds **1**, **2** and **5** performed very well against this pathogen, out-performing fenpropimorph in each case. The 5-membered ring compound, **11**, also performed well, but the 7-membered ring compound, 8, was inactive. The behaviour of the ring methyl-substituted materials was curious. Both the salts **10** and **7** failed to give control at 400 mg litre^{-1}. **6**, the free base of **7**, however, gave excellent control down to 10 mg litre^{-1}. This difference in activity has yet to be explained.

The overall best performers against both pathogens were the lead compounds **1** and **2**, although **6** showed almost equivalent activity.

4.4.3 Activity v. ergosterol biosynthesis (whole-cell assay)

All compounds exhibited activity in the micromolar range and some correlation between in-vivo activity against *E. graminis* and enzymatic activity can be observed (Table 1). Thus, the two least active compounds in the glasshouse, **4** and **11**, both have poor activity against the enzymatic pathway.

4.5 Series 2—Side-chain variation

4.5.1 Activity v. E. graminis

The underlying pattern behind the protectant and eradicant data for *E. graminis* f. sp. *hordei*, is not completely clear (Table 2). Changing the chain length appears to change activity in a manner that is dependent on whether the chain is substituted or not. The simplest benzyl analogue, **12**, is moderately active but loses this activity when a methyl group is substituted α to nitrogen as in **13** (see also **17**). **14** and **18**, the unsubstituted phenethyl analogues, are not very active, whereas **1**, with a methyl substituted α to nitrogen, is highly active. The unsubstituted phenylpropyl derivative, **16**, however, shows good activity, especially in comparison with **14**. The ethyl substituted compound, **15**, shows only moderate activity, indicating that there is probably a restriction on the size of the side-chain substituent for good activity.

In both eradicant and protectant tests v. *E. graminis* only the lead compounds, **1** and **2** performed at the same level as fenpropimorph.

4.5.2 Activity v P. recondita

Reasonable activity against *P. recondita* was observed with five compounds (Table 2). Four of these had methyl substitution α to the exocyclic nitrogen, whereas **12** and **14**, which were both without methyl substitution, were inactive. All five compounds performed either equivalently or in a superior fashion to fenpropimorph, but again no compound superior to either of the two lead compounds was uncovered.

4.5.3 Activity v. ergosterol biosynthesis (whole-cell assay)

A reasonable correlation between activity against *E. graminis* f. sp. *hordei* and suppression of ergosterol biosynthesis was again observed, with the least active compounds all registering inactive in the assay. Poorer correlation with *P. recondita* activity was noted.

4.6 Series 3—Phenyl substitution

4.6.1 Activity v. E. graminis *f. sp.* hordei

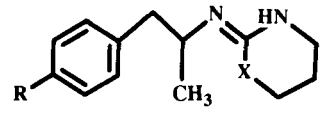
The protectant and eradicant data v. *E. graminis* f. sp. *hordei* show a clear pattern of activity (Table 3). This is especially true when the series 4-C_2H_5, 4-$CH(CH_3)_2$, 4-$C(CH_3)_3$, 4-$Si(CH_3)_3$ and 4-$Si(C_2H_5)(CH_3)_2$ is considered. For both the amidine and the guanidine series the ranking in activity is the same, 4-C_2H_5 < 4-$CH(CH_3)_2$ ≦ 4-$Si(CH_3)_3$ ≦ 4-$C(CH_3)_3$ < 4-$Si(C_2H_5)(CH_3)_2$. This is best exemplified in Table 5, in which LD_{50} concentrations calculated from a single comparative test against *E. graminis* v. sp. *hordei*, are tabulated.

When larger substituents than ethyltrimethylsilyl are used, protectant activity is lost. Thus the 4-C_6H_{13} (**21**, **22**), 4-$C_6H_5CH_2CH_2$ (**28**) and 4-naphthyl (**40**, **41**) substituted analogues are all inactive as protectants and the 4-C_6H_5 (**25**, **38**), 4-C_4H_9O (**24**, **37**) and 4-C_6H_5 (**27**) substituted analogues only lead to moderate activity. The 3-C_6H_5O analogues (**26**, **39**), the only examples chosen of 3-substitution, were inactive. Interestingly, despite their poor protectant activity, **37** (C_4H_9O) and **40** (4-naphthyl) showed good to excellent eradicant activity. As a general rule, little difference in activity could be distinguished between pairs of guanidine/amidine analogues similarly substituted. Two compounds showed activity clearly superior to the lead compounds **1** and **2**. These were the ethyldimethylsilyl amidines **35** and **36**.

4.6.2 Activity v. P. recondita

Unlike for series 1 and 2, similarity can be observed between the pattern of activity against *P. recondita* and that against *E. graminis* (Table 3). The compounds most active against one pathogen are also the most active against the other. Thus many of the analogues with bulky substituents, 4-naphthyl, 3-C_6H_5O, 4-OC_4H_9, 4-$C_6H_5CH_2CH_2$ for example, are inactive or have mediocre activity. The best activity is again observed in

TABLE 5
Effect of Size of Tail Substituent on Activity



Compound	R	X	LD_{50} (mg litre^{-1})[a] E. graminis hordei Prot.	Erad.
29	C_2H_5	CH_2	>400	NT
19	C_2H_5	NH.HCl	70	NT
31	$CH(CH_3)_2$	CH_2	20	10
20	$CH(CH_2)_2$	NH.HCl	10	NT
5	$C(CH_3)_3$	CH_2	8	10
1	$C(CH_3)_3$	NH.HCl	20	NT
23	$(CH_3)_3Si$	CH_2	8	5
22	$(CH_3)_3Si$	NH.HCl	20	NT
35	$C_2H_5Si(CH_3)_2$	CH_2	2	NT
23	$C_2H_5Si(CH_3)_2$	NH.HCl	6	NT

[a] NT = Not tested.

the lead compounds and the 4-trimethylsilyl and ethyldimethylsilyl analogues.

4.6.3 Activity v. ergosterol biosynthesis (whole-cell assay)

A definite correlation between in-vivo activity against both pathogens and suppression of ergosterol biosynthesis was again observed (Table 3). Compounds poorly active in vivo usually registered as inactive in the assay. Almost all the compounds most active *in vivo* exhibited a similar level of potency, at the enzyme level, to the two lead compounds **1** and **2**.

Three compounds, **21**, **28** and **37**, affected overall radiolabel incorporation into sterols without affecting the sterol profile. One reason for this could be that the compounds are inhibiting steps in the ergosterol biosynthesis, pre-squalene. However, as the assay is a whole-cell assay, it is also possible that there is interference with alternative biochemical processes.

5 CONCLUSIONS

Consideration of the mechanisms of both the Δ^{14} reductase and Δ^8–Δ^7-isomerase enzymes and the mode of action of known, fungicidal inhibitors of these enzymes, in particular the morpholine/piperidine class of fungicides, has led to the design of a novel class of compounds aimed at this mode of action. Pioneer examples have been synthesised and have been demonstrated to be good inhibitors of ergosterol biosynthesis in *U. maydis*. They also exhibited excellent fungicidal activity against *E. graminis* f. sp. *hordei* and *P. recondita* in in-vivo glasshouse tests.

Using these compounds as starting points, a systematic structural variation has been carried out. Testing a wide range of analogues at high volume confirmed the potential of this class of compound to control mildew and rust pathogens at levels comparable to those of the standards. Correlation of in-vivo and enzymatic data is good and the structure–activity relationship developed for this series of compounds closely parallels that found for the morpholine/piperidine class of fungicides, suggesting a common mode of action for the two classes.

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