Journal of Chromatography, 469 (1989) 293–304
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 21 341

SEPARATION OF FUNGAL STEROLS BY NORMAL-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

APPLICATION TO THE EVALUATION OF ERGOSTEROL BIOSYNTHESIS INHIBITORS

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(First received September 9th, 1988; revised manuscript received January 24th, 1989)

SUMMARY

An isocratic normal-phase high-performance liquid chromatographic method is reported which can separate radio-labelled sterols produced *in vitro* by cell-free extracts of the phytopathogenic fungus, *Ustilago maydis*. The method is rapid with typical analysis times of 15–20 min. It allows a reproducible separation of at least 7 radio-labelled sterols which have been identified by gas chromatography–mass spectrometry and enzyme inhibitor studies. This method can be used to obtain quantitative data on the inhibitory action of potential fungicides that disrupt ergosterol synthesis, and in favourable cases, the precise enzyme involved.

INTRODUCTION

Sterols are an important constituent in fungal cell membranes. Ergosterol is the predominant fungal sterol, even though it is not synthesised by some fungi such as the *Pythiaceae* family[1]. This is in contrast to plant and mammalian membranes in which C-24 alkylated sterols and cholesterol are the predominant sterols respectively[2].

Ergosterol is present in the phospholipid bilayer of the fungal membrane. Because of its structure and amphiphilic nature, ergosterol is thought to increase the membrane microviscosity; *cf.* cholesterol in mammalian systems[3]. This property alters the fluidity and molecular motion of other lipids[4], which may in turn modulate the activity of membrane-bound enzymes such as chitin synthetase and ATPase's[5]. Thus, ergosterol biosynthesis is essential for maintenance of normal growth and physiological status of the fungal cell.

An important and growing class of fungicides owe their fungitoxity to the inhibition of ergosterol biosynthesis. Examples of such compounds include the triazoles[6], pyrimidines[7] and phenylpropylamines[8].

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0021-9673/89/$03.50

Researchers are increasingly interested in the qualitative and quantitative aspects of enzyme inhibition by antifungal compounds. This has led to the development of chromatographic methods capable of separating sterol intermediates and ergosterol.

Early methods used conventional silicic acid column chromatography[9], but only partial resolution of some sterol intermediates was achieved with retention times of 2–3 days. Thin-layer chromatography (TLC) has also been extensively employed[10], although it suffers from certain disadvantages such as low loading capacities, relatively low resolution, and difficulty in the recovery of the purified compounds.

Gas-liquid chromatography (GLC) has been widely used over the last two decades in the analysis of free sterols and their derivatized esters[11]. Patterson[12], investigated structure–retention relationships of sterols in GLC. The great advantage of GLC over other chromatographic techniques is that it is ideal for interfacing with mass spectrometry (MS)[13], giving highly specific and sensitive detection coupled with structural information.

However, there are disadvantages of GLC such as: many sterols are thermally unstable, sample recovery is difficult or impossible, where sample recovery is possible the low column loading prohibits preparative separations, and detection of radiolabelled sterols is difficult to achieve.

High-performance liquid chromatography (HPLC) has inherent advantages over TLC and GLC; chromatographic separation times are usually measured in minutes, HPLC is a non-destructive technique allowing easy collection of separated analytes, and it has greater potential for accomplishing difficult separations because it can utilize a larger range of separation mechanisms, *viz.* absorption, partition, reversed-phase partition, chemisorption, gel permeation, ion exchange and ion-pair formation. The application of HPLC to the analysis of sterols has increased over the last ten years, with most workers employing reversed-phase systems (*e.g.* ref. 14). Where radiolabelled sterols have been separated, the radioactivity was determined by eluent fraction collection and subsequent liquid scintillation counting.

Hansbury and Scallen[15] reported a three step procedure for separating complex mixtures of sterol intermediates in cholesterol biosynthesis in which both reversed-phase HPLC and normal-phase HPLC were utilised. Evershed *et al.*[16] commented that the problem with HPLC was the lack of resolution of different steryl esters, making it difficult to assign unambiguous identifications of components in complex mixtures; the lack of a strong chromophore meant that the UV detection of steryl esters have relatively poor detection limits, requiring 10–50 mg of each component for analysis. The majority of sterol separations by HPLC have been applied to sterols occuring in mammalian systems, *e.g.* cholesterol and its precursors. An HPLC method describing the separation and detection of squalene, 2,3-epoxysqualene, lanosterol and other sterols in the ergosterol biosynthesis pathway has not been reported.

In this study we report an isocratic normal-phase HPLC system using an on-line radiochemical HPLC detector. It is a rapid, sensitive method capable of separating and quantifying radioactive non-saponifiable lipids formed from cell-free extracts of the phytopathogenic fungus *Ustilago maydis*. This technique was used to determine the activity of commercial ergosterol biosynthesis inhibitors.

EXPERIMENTAL

Apparatus

High-performance liquid chromatography. The chromatography was performed with an HPLC system consisting of two high-pressure pumps (Models 510 and 600A; Waters Assoc., Milford, MA, U.S.A.), an automatic sample injector (WISP; Waters Assoc.) and a radioactivity HPLC monitor (Model LB 506C; Berthold U.K., Leeds, U.K.). For spectrophotometric detection a variable-wavelength UV detector (Model LC55, Perkin-Elmer, Beaconsfield, U.K.) was used. The HPLC column was stainless steel (250 × 4.6 mm) packed with Hypersil 3 μm silica (Hichrom, Reading, U.K.) and heated to 35°C for all analyses. A stainless-steel guard column (10 × 4.6 mm) packed with Hypersil 5 μm silica was used as an on-line filter.

Radioactivity detector. A Berthold LB506C radioactivity chromatography system was used to measure the radiolabelled sterols separated by HPLC. The high-energy window channel was employed to measure carbon-14 with a liquid flow-through cell (0.5 ml) used throughout. The counting efficiency was typically 40-50%, as determined by liquid scintillation counting in a Beckman (Fullerton, CA, U.S.A.) LS1800 counter, and the cpm were converted into dpm using the following formulae: dpm = counts × total flow-rate through the cell (ml/min)/counting efficiency × active cell volume (ml).

Gas chromatography-mass spectrometry. Samples for mass analysis were produced by parallel enzyme assays using DL-mevalonic acid in one assay, and DL-[2-^{14}C]-mevalonic acid in the other. The radiochromatogram of the ^{14}C-labelled sterols was used to determine the areas of peak collection for the unlabelled eluent. Each sample was evaporated to dryness under nitrogen before conversion to the trimethylsilyl (TMS) derivatives prior to mass analysis. The TMS derivatives were formed by adding pyridine (50-μl) and bis(trimethylsilyl)trifluoroacetamide (BSTFA) (199 μl) before heating the sealed tube for 30 min at 60°C.

The derivatized samples were separated and analysed in a Finnigan 1020 automated GC–MS system (incorporating a Data General Nova 3 computer); the GC system was fitted with a 30 m × 0.32 mm I.D. J & W Scientific silica column coated with 0.25-μm DB-1, and a splitless injector with a flush 30 s after sample injection to remove residual gases. The end of the column was introduced directly into the mass spectrometer analyser chamber. The system was operated under the following conditions: helium pressure 11 lbs./in.^{2}; injector temperature 300°C; GC temperature 75–300°C at 3°C/min. The mass spectrometer was set to scan 40–650 a.m.u. per nominal second with an ionizing voltage of 70 eV. The filament was switched on 250 s after injection of the sample into the gas chromatograph.

Chemicals and reagents

Dichloromethane, *n*-hexane and methanol were of HPLC grade (Romil Chemicals, Loughborough, U.K.), magnesium chloride ($MgCl_2$), manganese chloride ($MnCl_2$), potassium hydroxide (KOH), and pyridine were analytical grade. The following chemicals, co-factors, buffers and HPLC standards were all obtained from Sigma, Poole, U.K., BSTFA + 1% trimethylchlorosilane (TMCS), flavin adenine dinucleotide (FAD), reduced nicotinamide adenine dinucleotide phosphate (NADPH), S-adenosyl methionine *p*-toluenesulphonate (SAM), adenosine triphos-

phate (ATP), ethylenediaminetetraacetic acid (EDTA), N-2-hydroxyethylpiperazine-N-2-ethanesulphonic acid (HEPES), phenylmethyl sulphonyl fluoride (PMSF), dithiothreitol (DTT), squalene, lanosterol and ergosterol. DL-[2-^{14}C]mevalonic acid DBED (N,N-dibenzylethylenediamine salt) (48.6 mCi/mmol) was obtained from DuPont (U.K.), New Research Products, Stevenage, U.K.

Tolnaftate, prochloraz and fenpropimorph were obtained from Sandoz, Vienna, Austria, Schering Agrochemicals, Saffron Walden, U.K. and Dr. Maag, 8157 Dielsdorf, Switzerland, respectively.

All aqueous solutions of co-factors and reagents were prepared in double-distilled water. The scintillation fluid used was Beckman Readysafe liquid scintillation cocktail.

Biosynthesis and extraction of radiolabelled compounds

Fungal cultures. Sporidia of *Ustilago maydis* (103760:CMI, 1963) were maintained on potato dextrose agar (Oxoid, CM139) slopes. Liquid cultures (1 l) of *U. maydis* in 2.8-l Erlenmyer flasks were grown in YED media (1.5% D-glucose, 0.3% yeast extract) and shaken in a rotary incubator at 120 rev./min at 25°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 media at 4°C.

Preparation of the cell-free enzyme system. The washed cells were suspended in 25 ml of homogenisation media (50 m*M* HEPES pH 7.4, 1 m*M* EDTA, 5 m*M* DTT and 10 mg/100 ml each of soybean trypsin inhibitor and PMSF), and an equal volume of glass beads, 0.2 mm diameter. The cells were then disrupted by two 45-s periods of homogenisation at 4°C in a bead beater (Biospec Products). The resultant homogenate was centrifuged at 10 000 *g* for 15 min and the supernatant (S_{10}) used for enzyme assays.

Enzymic assay. 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), 3 m*M* DL-[2-^{14}C]mevalonic acid (0.2 μCi/assay), 1.7 m*M* NAD, 3.5 m*M* ATP, 1.7 m*M* NADPH, 1.7 m*M* S-adenosyl-L-methionine, 0.17 m*M* FAD, 3.5 m*M* $MgCl_2$ and 3.5 m*M* $MnCl_2$ 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 15% KOH in ethanol (0.5 ml). The mixture was heated at 70°C for 30 min and then extracted with two 1-ml aliquots of *n*-hexane to remove the non-saponifiable lipids. The combined hexane extracts were then evaporated to dryness under nitrogen. The extracted lipids were re-dissolved in 200 μl of *n*-hexane prior to HPLC analysis.

Chromatographic procedure

Standard solutions of squalene, lanosterol and ergosterol in dichloromethane were found to give an acceptable UV absorption at 245 nm. In an effort to minimise extra-column band broadening effects the guard column was directly coupled to the head of the analytical column, and the connective stainless-steel tubing (1.5 mm × 0.13 mm I.D.) was kept as short as possible. Various mobile phases were tested and 0.025% methanol in dichloromethane was found to give the best separation in a reasonable time (15–20 min) for routine sample analysis. The samples in *n*-hexane (200 μl) were transferred to sealed autosampler vials fitted with limited volume inserts and screw tops with PTFE septa. A volume of 190 μl was taken by the auto sampler and injected onto the HPLC column.

When monitoring radiolabelled samples the on-line radioactivity detector was connected in place of the variable wavelength UV detector, via a mixing "T" block, into which the liquid scintillation cocktail was pumped at a rate of 3.5 ml/min. The stainless-steel connective tubing leading to the radioactivity detector was crimped every 6 mm to ensure complete mixing of the scintillant with the column eluent before entering the 0.5 ml flow-through cell.

All mobile phase combinations were mixed, passed through a 0.2-μm membrane filter (Anachem, Luton, U.K.) under vacuum and used immediately at a flow-rate of 1.0 ml/min. Chromatographic retention data were expressed as capacity ratios: $k' = (t_R - t_0)/t_0$, where t_R and t_0 are the retention times of the analyte and a non-retained compound respectively. The shape of each peak was assessed by measuring the peak asymmetry factor (AF). This was calculated by dropping a perpendicular from the peak maximum and measuring the distance from this line to the leading edge (a) and the trailing edge (b) at the 10% peak-height level ($AF = b/a$).

Statistical treatment of results

Results are expressed as mean $\pm$ S.E.M. with the number of replicate experiments given in parentheses. Radiochromatograms are from a typical experiment performed in duplicate.

RESULTS AND DISCUSSION

Although a high proportion of the literature methods employ reversed-phase systems, the lipophilicity of the sterols under investigation suggested that a normal-

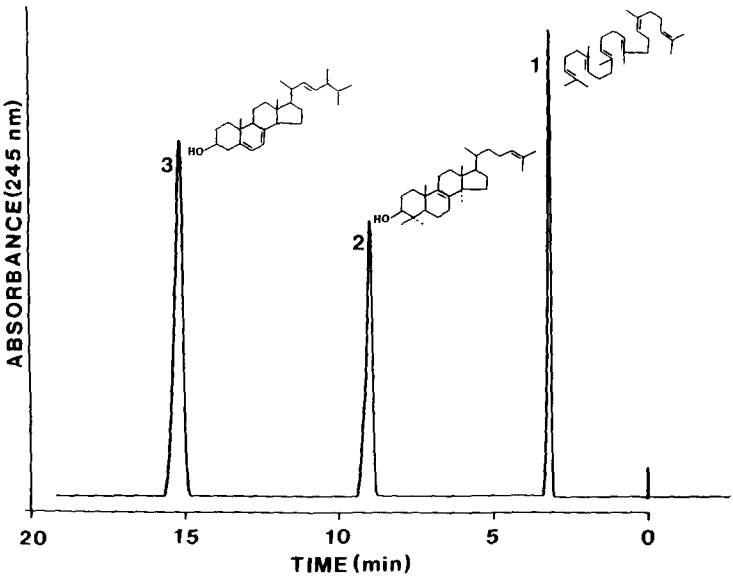

Fig. 1. HPLC separation of a mixture of (1) squalene (2 g), (2) lanosterol (0.8 g) and (3) ergosterol (0.8 g). The column (25 cm × 4.6 mm I.D.) contained 3 μm Hypersil silica and the mobile phase was 0.025% methanol in dichloromethane at 1 ml/min. Detection was by UV using a Perkin-Elmer LC55 detector at 245 nm.

phase system might be more efficient. Method development was carried out using standard solutions of squalene, lanosterol and ergosterol, with UV detection at a compromise wavelength of 245 nm. Note that squalene is virtually unretained under the conditions used to obtain the chromatogram shown in Fig. 1. Although this could be thought to be undesirable (as all retained solutes would elute at this point in the chromatogram), this does not cause any problems in practice: MS showed that the 0–4 min portion of the eluent collected after injection of authentic samples contained mobile phase and squalene only. Good separation of squalene, lanosterol and ergosterol (see Fig. 1) was achieved using a mobile phase of dichloromethane with 0.025% methanol and was therefore used throughout this study. The ergosterol peak symmetry shown in Fig. 1 demonstrates reasonable column performance for the longest retained peaks under the chromatographic conditions chosen.

A cell-free extract of *U. maydis* converted DL-[2-^{14}C]mevalonic acid into non-saponifiable lipids as previously reported for *Saccharomyces cerevisiae*[17,18]. However, previous studies with cell-free biosynthetic systems have separated and identified few sterols. The methods used have been either GLC[17] or TLC[18]. In the former study [17] only two peaks were identified by radio-GC whilst five radioactive peaks were found using silicic acid column chromatography. The latter study of Gadher *et al.*[18] was able to show the TLC separation of squalene, 2,3-epoxysqualene and three classes of sterols; 4,4-dimethylsterols, 4α-methylsterols and 4-desmethylsterols. The normal-phase HPLC method reported herein can resolve squalene, 2,3-epoxysqualene and at least 9 radio-labelled peaks, of which 7 have been identified as sterols (see Fig. 2). The peak identities in Table II were assigned in the following way; peaks 1, 2, 4, 5, 7 and 8 gave mass spectra that are identical to reference spectra from authentic samples. Peaks 3 and 6 were identified by mass fragmentation analysis and will be reported elsewhere.

Interestingly, this method has resulted in the identification of a relatively pure peak of radio-labelled ergosterol; co-crystallization of this peak (t_R = 15 min 18 s)

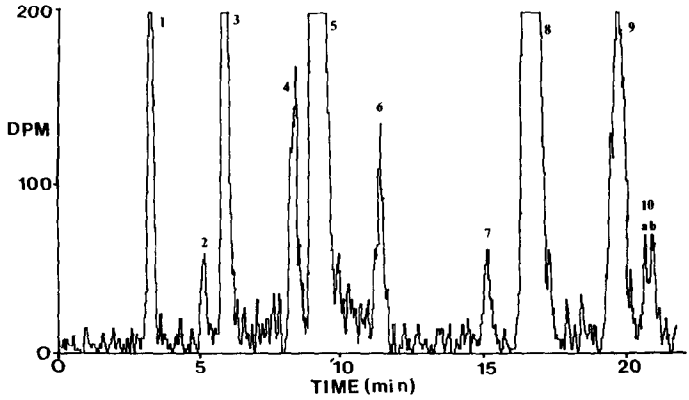

Fig. 2. A typical radiochromatogram of the ^{14}C-labelled non-saponified lipids formed from incubating [^{14}C]mevalonic acid with cell-free extracts of *U. maydis*. The peak identities are given in Table II. Chromatographic conditions were as in Fig. 1, except for the substitution of a Berthold LB 506C HPLC radiomonitor as the detector.

TABLE I

CHROMATOGRAPHIC DATA FOR A 3-μm HYPERSIL COLUMN WHEN USED WITH SQUALENE, LANOSTEROL AND ERGOSTEROL

Chromatographic conditions: a stainless-steel column (25 cm × 4.6 mm I.D. packed with 3-μm Hypersil silica) was used with a mobile phase of 0.025% methanol in dichloromethane at a flow-rate of 1 ml/min. A Perkin-Elmer LC55 UV detector was used at 245 nm. t_R = retention time, k = capacity factor, N = number of theoretical plates, AF = assymetry factor and R_s = resolution. t_o = 2.7 min.

Solute	*Parameter*				
	t_R	k'	N	AF	R_S
Squalene	3.1	0.15	41	1.2	
					19.7
Lanosterol	9.0	2.33	5208	1.5	
					13.8
Ergosterol	15.2	4.6	8264	2.0	

with authentic ergosterol gave a constant specific activity at approximately 75% of the initial radioactivity (Table III). The implications of the complete conversion of [^{14}C]mevalonate to [^{14}C]ergosterol with regard to the preferred sterol biosynthetic pathway in *U. maydis* will be discussed in a further report. The proposed biochemical pathway from squalene to ergosterol in fungi has been recently reviewed by Mercer[19].

Of the total radioactivity added as [^{14}C]mevalonic acid, 33 ± 4% (n=6) was recovered in the non-saponifiable extract, indicating good conversion of radiolabel into sterols. Indeed this figure is an underestimate of the true conversion of radiolabel into sterol because only the L-isomer and not the D-isomer of mevalonic acid is the natural precursor for sterol synthesis. Thus since the racemate DL-[2-^{14}C]mevalonic

TABLE II

MS ANALYSIS OF THE HPLC RADIO-LABELLED PEAKS IN FIG. 3

Radio-labelled peak	*Compounds identified*	t_R *(HPLC) (min)*	*m/e*
1	Squalene	3.60	410
2	2,3-epoxysqualene	5.13	426
3	4,4-dimethylergosta-8-ene-3β-ol	6.3	428
4	24-methylene-24,25 dihydrolanosterol	8.5	440
5	lanosterol	9.5	426
6	4-methylergosta-8,24(28)-diene	11.3	412
7	Ergosterol	15.3	396
8	Fecosterol	16.6	398
	Episterol	16.6	398
9	ND[a]	20.3	–
10a	ND	21.1	–
10b	ND	21.3	–

[a] ND = not determined.

TABLE III

IDENTIFICATION OF RADIOLABELLED ERGOSTEROL IN THE HPLC PEAK AT t_R 15 min 18 s, BY INVERSE ISOTOPE DILUTION

50 mg of authentic ergosterol was added to a sample of peak t_R 15 min 18 s, dissolved in acetone and crystallised from acetone/water.

Number of crystallisations with authentic ergosterol	*Specific activity (dpm/mg)*
0	3096
1	2633
2	2345
3	2332

acid was used in this study a radiolabelled conversion of 66% has probably been achieved. The rate of synthesis of ergosterol from [^{14}C]mevalonic acid *in vitro* is 0.020 $\pm$ 0.014 ($n = 5$) nmol/mg protein/h (assuming 5 moles of ^{14}C are incorporated into 1 mole of ergosterol with the loss of one ^{14}C as $^{14}CO_2$ in a C-4 demethylation step and 75% of the "ergosterol" peak t_r = 15 min 18 s, is authentic ergosterol). The specific rate of ergosterol synthesis, reported in this study, is similar to that of sterol synthesis found in mammalian systems, *e.g.* cholesterol synthesis in rat liver peroxisomes and microsomes is 0.087 and 0.135 nmol/mg protein/h respectively[20].

It should be noted however, that sporidial cultures of *U. maydis* greater than 24 h rapidly lose the ability to synthesise ergosterol and even episterol and other C-4 desmethyl sterols. For example, a 10 000 *g* supernatant fraction from a ten day old culture of *U. maydis* gave 90% of non-saponifiable fraction as squalene with the remaining 10% being lanosterol[21]. No other radiolabelled sterols were detected. The ability to synthesise ergosterol appears to be synchronized with the metabolically active growing phase of the fungi.

The results of the inhibitor studies support the proposed identity of the radiolabelled sterols. Thus, tolnaftate, which inhibits the enzyme squalene monoxygenase[22] abolished most of the sterols at a concentration of 50 μM (Fig. 3a). The squalene peak is increased from 2% of the non-saponified lipids to 89.5% (Table IV) and the lanosterol peak is reduced form 58.5% to 8.3%. The peak at t_R = 16.4 min represents less than 2% of the radioactivity in the non-saponified extract and was not further characterized. The percentage radioactivity recovered in the non-saponified extract is decreased from 33 to 21.2%. This decrease, in the presence of tolnaftate, has been noted several times and may suggest that a regulatory feedback mechanism may exist when squalene concentrations are increased to a particular level or alternatively tolnaftate, at 50 μM, partially inhibits an earlier step in squalene synthesis. Further experiments are needed to support the above suggestions. However, this result shows that tolnaftate can inhibit squalene monoxygenase from a phytopathogenic fungus, albeit at a higher concentration compared to the inhibition of squalene monoxygenase in a mycopathogenic fungus such as *Candida albicans*[22].

The fungicide prochloraz, which is a C-14 demethylase inhibitor, completely abolished the ergosterol and fecosterol/episterol peaks at a concentration of 10 μM (Fig. 3b). A large increase in squalene and a relatively small increase in lanosterol is

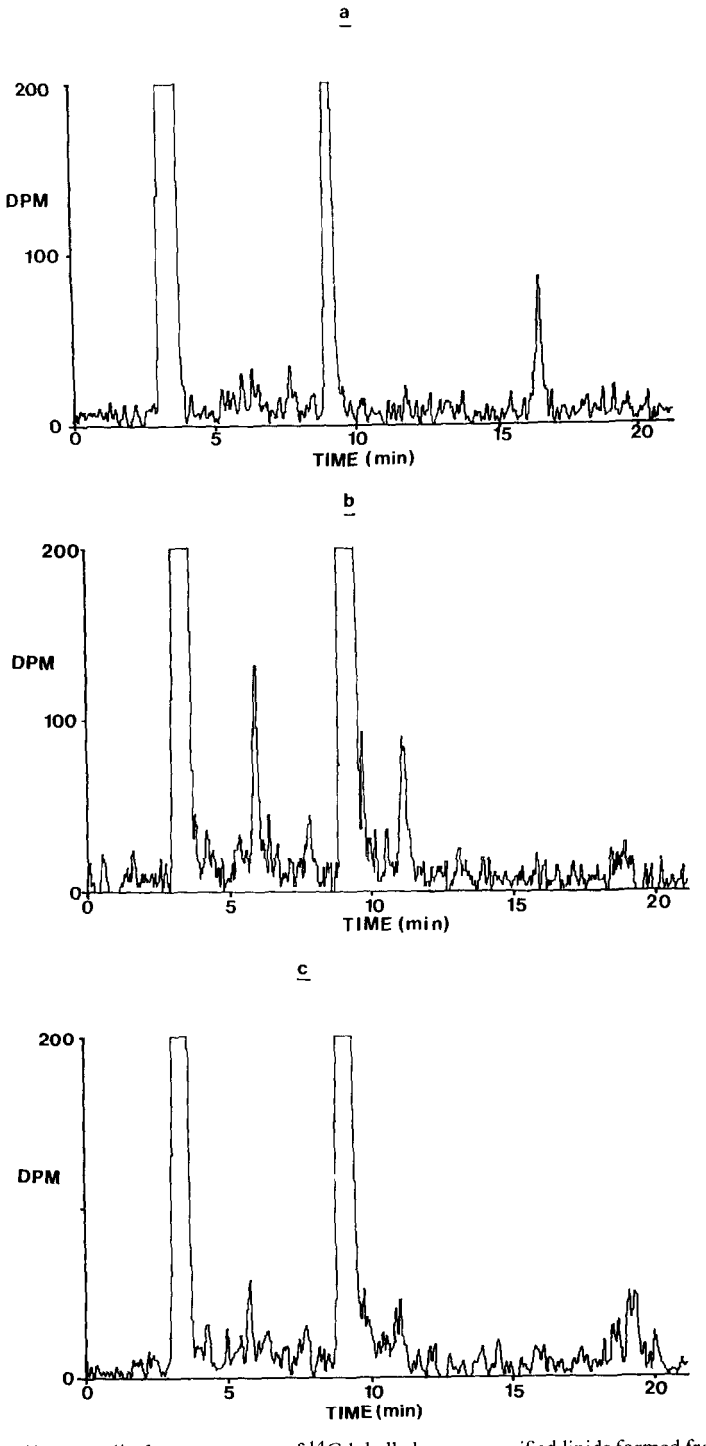

Fig. 3. Radiochromatograms of ^{14}C-labelled non-saponified lipids formed from incubating [^{14}C]mevalonic acid with cell-free extracts of *U. maydis* in the presence of (a) 50 μM tolnaftate, (b) 10 μM prochloraz and (c) 10 μM fenpropimorph.

TABLE IV

EFFECT OF 3 ANTIFUNGAL COMPOUNDS ON THE CONVERSION OF DL-[2-^{14}C]MEVALONIC ACID TO NON-SAPONIFIED LIPIDS

The *in vitro* enzymic assay was as described in the experimental section but included 5 μl of the test compound in acetone. Acetone (5 μl) alone, had no effect on the incorporation of radioactivity into the non-saponified lipids.

Treatment	*% Radioactivity recovered in non-saponified extract*[a]	*Distribution of radioactivity in the non-saponified extract*[b] *(%)*		
		Squalene	*Lanosterol*	*Episterol/ fecosterol*
Control	33.0	2.0	58.5	25.3
Tolnaftate (50 μM)	21.2	89.5	8.3	0
Prochloraz (10 μM)	32.6	32.0	65.2	0
Fenpropimorph (10 μM)	28.3	25.1	73.2	0

[a] The figures represent the % radioactivity recovered in the non-saponified extract compared to the total radioactivity added as DL-[2-^{14}C]mevalonic acid.

[b] The figures are of % radioactivity in the respective sterol fractions compared to the total radioactivity recovered in the non-saponified extract.

observed (See Table IV). The 4,4-dimethyl-5α-ergosta-8-ene-3-β-ol and 24-methylene-24,25-dihydrolanosterol peaks were reduced by 45 and 37% respectively. Although this altered profile supports the view that the primary mode of action of prochloraz is inhibition of the C-14 demethylation of 24-methylene-24,25-dihydrolanosterol (or lanosterol in yeast) at the concentration used in this study (10 μM), partial inhibition of the C-24 methylation of lanosterol may occur.

Fenpropimorph gave similar results (see Fig. 3c). The ergosterol and fecosterol/episterol peaks were completely abolished. The squalene and lanosterol peaks are similarly increased as seen with prochloraz (Table IV). The 4,4-dimethyl-5α-ergosta-8-ene-3-β-ol and 24-methylene-24,25-dihydrolanosterol peaks decreased by 80% and 50% respectively.

The interpretation of this altered profile is not straightforward; at a concentration of 10 μM fenpropimorph may have inhibited the Δ^{14}-reductase step, which appears to reduce 4,4-dimethyl-5α-ergosta-8,14-diene-3β-ol to 4,4-dimethyl-5α-ergosta-8-ene-3β-ol in our system, since the latter sterol was almost abolished. This would be in agreement with the work of Baloch and Mercer[23] who showed that in *Saccharomyces cerevisiae*, fenpropimorph inhibited the Δ^{14}-reductase step at high concentrations (IC_{50} = 2.3 μM) whereas at lower concentrations (IC_{50} = 13 nM) was preferentially inhibited. However this interpretation of the altered sterol profile should be cautioned since there was no evidence for the accumulation of $\Delta^{8,14}$- or Δ^{8}-sterols, which does occur in fungal cellular liquid cultures when treated with fenpropimorph[24]. In the cell-free system in this study, the accumulation of a $\Delta^{8,14}$-sterol may have occurred but was not resolved or detected under the experimental conditions used.

Thus although the altered sterol profiles support the proposed modes of action

of the three fungicides tested at a single dose in this study, more informative data can be achieved by testing compounds in a dose–response manner. Consequently the enzyme that is exquisitely sensitive to inhibition may be identified by the sequential return of sterol peak(s) to control levels. This enables IC_{50} values (the concentration at which a known sterol peak is reduced by 50%) to be calculated for a given compound *e.g.* tolnaftate has an $IC_{50} = 4\ \mu M$ for inhibition of lanosterol synthesis and fenpropimorph and $IC_{50} = 220$ nM for inhibition of episterol synthesis in *U. maydis*[21]. Although these values cannot be regarded as"absolute" IC_{50} values for the enzymes responsible for synthesizing lanosterol and episterol, they can be used to determine relative potencies for a series of structurally-related compounds. The precise enzyme inhibited, however, can only be definitively proven by single enzyme studies, yielding "accurate" K_i inhibition constants and the type of inhibition involved.

CONCLUSION

In conclusion, the isocratic normal-phase HPLC method reported here can separate radio-labelled sterols produced in cell-free extracts of *U. maydis* in a rapid and reproducible manner. This method can be used to obtain quantitative data on the inhibitory action of potential fungicides that disrupt ergosterol biosynthesis. The enzyme(s) involved in the inhibition may also be identified by observing the altered sterol profiles, although it is accepted that single enzyme studies are required to ultimately prove specific enzyme inhibition. This HPLC system is currently being used to clarify and provide further experimental evidence for the proposed sterol biosynthetic pathway in phytopathogenic fungi.

ACKNOWLEDGEMENTS

The authors would like to thank Dr. W. Greenaway, Department of Plant Sciences, University of Oxford, for providing GC–MS analysis and Drs. M. Jung, P. F. S. Street and R. Baloch (Dow Chemical Co. Ltd., Letcombe Laboratories) for helpful discussions at various times throughout this study.

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