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Radio-detection high-performance liquid chromatographic enzyme assay for inhibitors of fungal sterol Δ^{14}-reductase

CHRISTOPHER C. STEEL[a]

Department of Agrobiological Research, Dr. R. Maag Ltd., CH-8157 Dielsdorf (Switzerland)

ABSTRACT

An enzyme assay for inhibitors of fungal sterol Δ^{14}-reductase employing isocratic reversed-phase high-performance liquid chromatography is described. A Hypersil 5-μm octadecylsilyl (ODS) column (250 mm × 4.6 mm I.D.) was used and a mobile phase consisting of methanol–water–ethanol (86:4:10, v/v) was pumped at a flow-rate of 1.5 ml/min. Typical analysis times were 15 min. Using [4-^{14}C]ignosterol as a substrate and an enzyme preparation from *Saccharomyces cerevisiae*, this method was used to compare the inhibition of sterol Δ^{14}-reductase by the fungicides fenpropidin and fenpropimorph with three N-substituted 8-azadecaline compounds.

INTRODUCTION

The fungal sterol biosynthetic pathway has been a focus for the development of fungicides and antimycotics for the past twenty years. Such antifungal compounds whose mode of action is dependent upon an inhibition of the enzymes involved in the biosynthesis of sterols are collectively referred to as sterol biosynthesis-inhibiting compouns or SBIs [1]. Inhibition of sterol biosynthesis in both fungal and plant cells results in an accumulation of structurally abnormal sterols in the cell membrane [2,3]. Such an accumulation results in a membrane with altered fluidity and permeability properties [4,5] and probably explains the ultimate cause of fungal death [6].

Sterol Δ^{14}-reductase is one of the target enzymes of a number of SBI fungicides and antimycotics [7,8]. The enzyme requires NADPH as a co-factor, has a pH optimum in the region of 6.0 and does not require the presence of oxygen [9]. It is responsible for the reduction of the $\Delta^{14\ (15)}$ double bond following the 14α-demethylation of either lanosterol to give 4,4-dimethyl-5α-cholesta-8,24-dien-3β-ol in the case of *Saccharomyces cerevisiae* or 24-methylene-24(25)-dihydrolanosterol to give 4,4-dimethyl-5α-ergosta-8,24(28)-dien-3β-ol in most other fungi [10]. However, fungi grown in the presence of inhibitors of sterol Δ^{14}-

[a] Present address: New South Wales Government, Department of Agriculture, Biological and Chemical Research Institute, Private Mail Bag No. 10, Rydalmere, N.S.W. 2116, Australia.

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reductase are still able to demethylate the sterol substrate, and so demethylated sterols such as 5α-ergosta-8,14,dien-3β-ol (ignosterol) accumulate rather than 4,4-dimethyl sterols [4,9]. When purified from inhibitor-grown fungi, ignosterol is able to function as a substrate for the sterol Δ^{14}-reductase enzyme [11].

Inhibitors of sterol Δ^{14}-reductase, such as 15-azasterol [9] and the fungicide fenpropimorph [11] are thought to act by mimicking the transition-state high-energy intermediate (HEI) and to bind to the enzyme more tightly than the natural substrate [6].

Previous reported methods for the assay of fungal sterol Δ^{14}-reductase involved prior derivatization of sterols by acetylation and separation of sterol substrate from sterol product by thin-layer chromatography (TLC) [9,11]. The development of a cell-free enzyme assay which avoids sample derivatization prior to chromatographic analysis and has a short analysis time is desirable for the rapid screening of candidate inhibitors of sterol Δ^{14}-reductase. Furthermore, a cell-free enzyme assay is the only direct evidence of inhibition of a given enzyme.

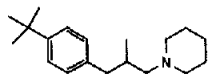
The use of a high-performance liquid chromatographic (HPLC) method for sterol analysis also has the advantage that the procedure is non-destructive allowing sample recovery for later analysis if required. The use of radiolabelled sterols further allows the specific detection of substrate and product in enzyme assays. Previous methods for the HPLC analysis of sterols have employed UV detection at 210 nm [4,12] which is not particularly suitable for enzyme assays when a crude enzyme preparation is used.

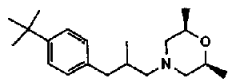
This paper reports on the use of a radio-detection HPLC enzyme assay for inhibitors of fungal sterol Δ^{14}-reductase employing a crude enzyme preparation from *S. cerevisiae* involving analysis times of 15 min. Using this method a series of three experimental N-substituted 8-azadecalines (Fig. 1) were compared with the fungicides fenpropidin and fenpropimorph as inhibitors of fungal sterol Δ^{14}-reductase.

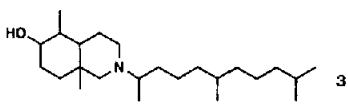
EXPERIMENTAL

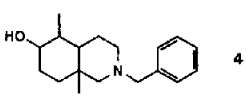
Chemicals and reagents

Yeast extract and tryptone for the growth of *S. cerevisiae* were obtained from Difco Labs. (Detroit, MI, U.S.A.). The following buffers, co-factors and standard chemicals of analytical grade were obtained from Fluka (Buchs, Switzerland): KOH, KH_2PO_4, nicotinamide, N-acetyl-L-cysteine, NADPH (reduced tetrasodium salt) and glucose-6-phosphate. Glucose-6-phosphate dehydrogenase (form baker's yeast) was obtained from Sigma (St. Louis, MO, U.S.A.). Methanol and ethanol were HPLC grade (Romil Chemicals, Loughbourgh, U.K.). Double-distilled water was used for the preparation of all buffers and HPLC solvents. When required [4-^{14}C]cholesterol (specific activity 142 μCi/mg) (Amersham Radiochemicals, Amersham, U.K.) was used as an internal standard. Fenpropidin (1) and fenpropimorph (2) (Dr. R. Maag, Dielsdorf, Switzerland) were









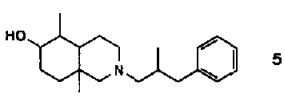


Fig. 1. Structures of inhibitors used in this study. 1 = Fenpropidin; 2 = fenpropimorph; 3 = N-(1,5,9-trimethyldecyl)-4α,10-dimethyl-8-aza-*trans*-decal-3β-ol; 4 = N-benzyl-8-aza-4α,10-dimethyl-*trans*-decal-3β-ol; 5 = N-[(3)-4-*tert*.-butylphenyl-(2-methyl)propyl]-8-aza-4α,10-dimethyl-*trans*-decal-3β-ol.

used as standard inhibitors in enzyme assays. Compounds 3, 4 and 5 were synthesized as described in refs. 13 and 14.

Ignosterol was chemically synthesized from ergosterol using the method of Dickson *et al.* [15]. [4-^{14}C]Ignosterol was prepared by first forming [4-^{14}C]ergosterol as described in refs. 16 and 17 and then transforming the radiolabelled ergosterol to [4-^{14}C]ignosterol as described in ref. 15 for unlabelled sterols.

Enzyme preparation

S. cerevisiae starter cultures were grown on the medium of Bottema and Parks [9] at 30°C aerobically using an orbital shaker (160 rpm) for 24 h as described previously [4]. Six infusion bottles containing 1.9 l of the same medium were each inoculated with 100 ml starter cultures and grown statically for 60 h. The cells were centrifuged at 4000 *g* for 10 min at 4°C and the pellet was washed twice with 0.1 *M* phosphate buffer (pH 6.2) supplemented with 30 m*M* nicotinamide and 5 m*M* N-acetyl-L-cysteine. The washed pellet was resuspended in the above buffer

to the extent of 0.75 ml/g fresh weight and the cells were homogenized in a Braun cell mill (cooled with CO_2) using equal volumes of cells to glass beads (diameters 0.25–0.30 mm and 0.17–0.18 mm in a ratio of 1:2). The cell fraction was decanted, filtered through glass wool and centrifuged at 8000 *g* for 20 min (4°C) to pellet the unbroken cells. The supernatant was freshly used as a cell-free enzyme preparation. The protein content of the preparation was determined according to the method of Bradford [18] and adjusted with phosphate buffer (pH 6.2) to give a protein concentration of 20 mg/ml.

Sterol Δ^{14}-reductase enzyme assay

Inhibitors were diluted in ethanol and delivered in 10-μl volumes to 30-ml pyrex tubes maintained on ice. [4-^{14}C]Ignosterol (specific activity 37.4 μCi/mg) was dissolved in ethanol and mixed with unlabelled ignosterol in a ratio of 13:87 and added to the enzyme preparation such that the final substrate concentration was 6.25 μM (0.5 μCi radiolabelled ignosterol per enzyme incubation). For enzyme kinetic studies, [4-^{14}C]cholesterol (0.5 μCi per sample), which was dissolved in ethanol, was used as an internal standard. The final concentration of ethanol in the enzyme incubations was 1.5% (v/v).

The enzyme preparation was supplemented with 1 mM NADPH (reduced tetrasodium salt) and an NADPH-regenerating system consisting of glucose-6-phosphate dehydrogenase (1 U/ml) and 10 mM glucose-6-phosphate. The enzyme suspension was then dispensed in 0.99-ml volumes to the tubes containing inhibitor at 4°C. The contents were mixed and the tubes incubated aerobically at 30°C using an orbital shaker (160 rpm). For the determination of IC_{50} (50% enzyme inhibition) values, the incubation period used was 60 min. At the end of the incubation period the reaction was terminated using 16% (w/v) KOH in 80% aqueous methanol (2 ml) and heating at 70°C for 1 h in a waterbath while shaking gently. After cooling, the non-saponifiable lipids were extracted with *n*-hexane (two 5-ml volumes) and the extracts taken to dryness. For HPLC analysis, the extracted lipids were dissolved in a 150-μl volume of methanol.

HPLC analysis

Sterols were analysed using a Varian Vista 5500 HPLC system linked to a Berthold LB506C radio-detector equipped with a Berthold GT-400 cell (400-μl volume packed with solid scintillator). The low-energy window channel was used to measure carbon-14 and the counting efficiency was 40%. Radiochromatograms were evaluated using a Berthold HPLC IBM computer programme. The HPLC column was a 5-μm Hypersil ODS column (250 mm × 4.6 mm I.D.) (Ercatech, Bern, Switzerland) operating at room temperature. The mobile phase used was a mixture of methanol–water–ethanol (86:4:10, v/v) which was pumped isocratically at a flow-rate of 1.5 ml/min. To avoid mixing problems the ethanol was added to the previously prepared aqueous methanol.

Calculation of sterol Δ^{14}-reductase inhibition

The inhibitor efficiency was calculated by comparing the percentage of substrate conversion in inhibitor incubations with that in a control incubation containing 10 μl of ethanol in the absence of inhibitor. The percentage of substrate conversion was calculated by dividing the dpm in the product peak by the total dpm and multiplying by 100. The percentage inhibition was then calculated from the expression $(C_{ct} - C_{inh}/C_{ct}) \times 100$ where C_{ct} and C_{inh} are the percentages of substrate conversion in control and inhibitor-containing incubations, respectively. The concentration of inhibitor required to give 50% enzyme inhibition, *i.e.* IC_{50}, was calculated using linear regression.

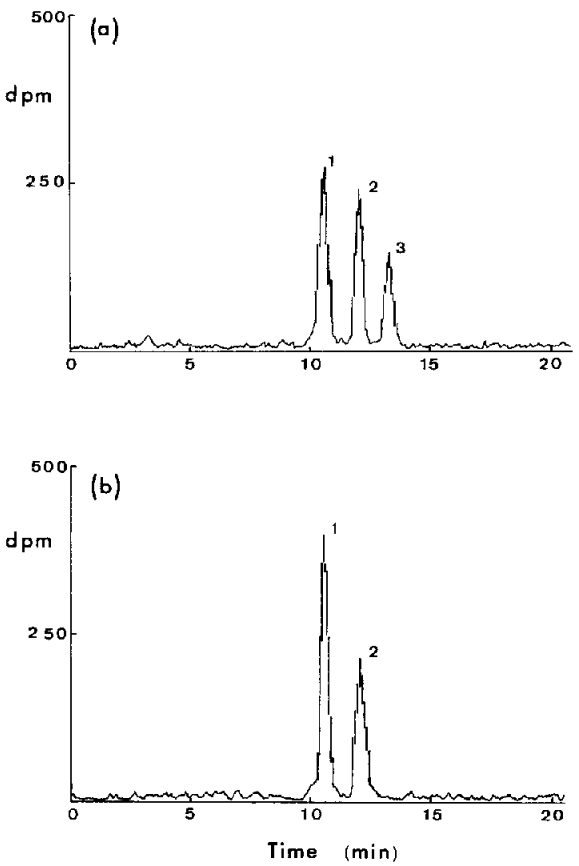


Fig. 2. Radiochromatograms of sterol extracts from typical enzyme incubations (1 h, 30°C) using [4-^{14}C]ignosterol as a substrate and [4-^{14}C]cholesterol as an internal standard. (a) Control incubation without inhibitor; (b) incubation with 10 μM fenpropimorph (compound 2). Peaks: 1 = ignosterol; 2 = cholesterol; 3 = reduction product of ignosterol.

RESULTS

Using the reversed-phase HPLC system described, [4-^{14}C]ignosterol and its reduction product were resolved with baseline separation (Fig. 2a). Ignosterol eluted in a volume of 15.8 ml and the product of the enzymatic reaction in 20.1 ml while cholesterol which was used as an internal standard eluted in a volume of 18.1 ml. The relative retention times of ignosterol and the reduced product with respect to cholesterol were 0.87 and 1.11, respectively. Fig. 2b is a radiochromatogram of a sterol extract from an enzyme incubation in the presence of 10 μM fenpropimorph which caused a total inhibition of the enzyme.

Formation of the product when ignosterol was incubated in the presence of an enzyme preparation from *S. cerevisiae* supplemented with NADPH and an NADPH-regenerating system was found to be linear for the first hour of incubation at 30°C (Fig. 3). After a longer incubation period the reaction rate eventually plateaued. No formation of product was detected when NADPH and the NADPH-regenerating system were omitted from the reaction mixture. Using a Linewaever–Burk plot, the appararent Michaelis constant (K_M) of the Δ^{14}-reductase for ignosterol was determined to be 5.7 μM and the maximum velocity of the reaction (V_{max}) was 0.83 nmol/min/mg protein (Fig. 4).

Using an incubation time of 60 min and a substrate concentration of 6.25 μM, the efficiencies of three N-substituted 8-azadecaline compounds were compared with those of fenpropidin and fenpropimorph as inhibitors of the sterol Δ^{14}-reductase enzyme.

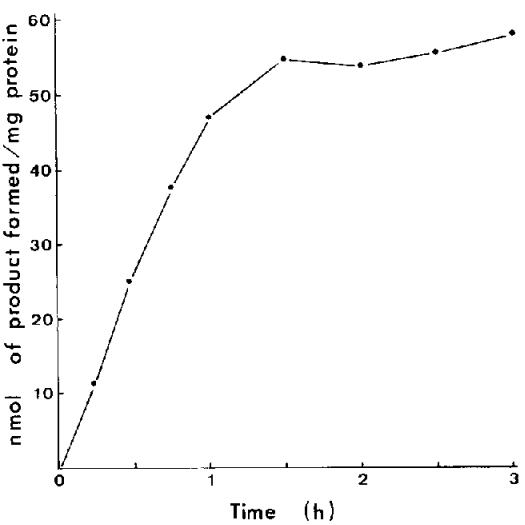


Fig. 3. Effect of incubation length on amount of product formed by sterol Δ^{14}-reductase. Substrate concentration was 6.25 μM and incubation temperature 30°C.

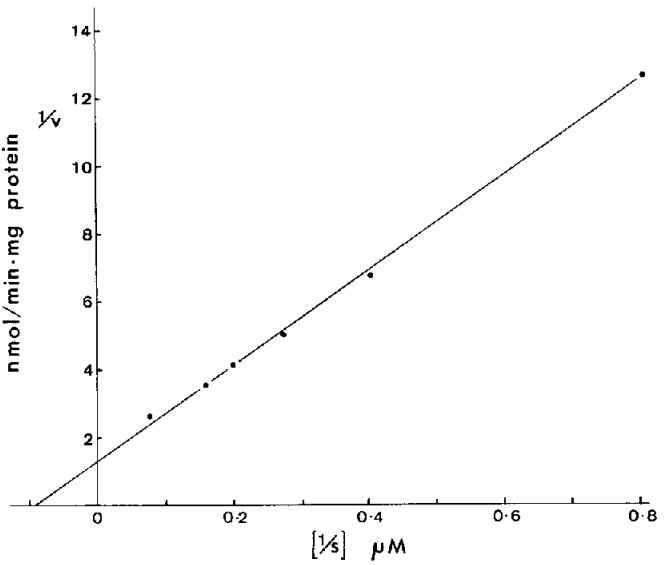


Fig. 4. Lineweaver–Burk double reciprocal plot for sterol Δ^{14}-reductase using ignosterol as a substrate. V_{max} = 0.83 nmol/min/mg protein and K_M = 5.7 μM.

Both fenpropidin (1) and fenpropimorph (2) were found to be inhibitors of sterol Δ^{14}-reductase which at 1.7 and 2.6 μM, respectively, inhibited the enzyme by 50% (*i.e.* IC_{50}) (Table I). However, two of the experimental compounds tested (3 and 5) were more efficient than either of the two standard inhibitors having IC_{50} values of 0.7 and 1.3 μM, respectively. Compound 4 was found to be only weakly active as an inhibitor of fungal sterol Δ^{14}-reductase.

TABLE I

INHIBITION OF Δ^{14}-REDUCTASE BY THE EXPERIMENTAL COMPOUNDS

The enzyme assay was carried out as described in the text using [4-^{14}C]ignosterol as a substrate (6.25 μM) and an incubation time of 1 h at 30°C.

Compound	Inhibition of Δ^{14}-reductase IC_{50} (μM)
1	1.7
2	2.6
3	0.7
4	95.4
5	1.3

DISCUSSION

The reversed-phase HPLC system used was clearly able to resolve ignosterol from the reduction product, *i.e.* 5α-ergosta-8-en-3β-ol. However, as the crude enzyme preparation from yeast presumably also contains the other enzymes of the sterol biosynthetic pathway, the 5α-ergosta-8-en-3β-ol formed is very likely converted by the sterol $\Delta^8 \rightarrow \Delta^7$-isomerase enzyme (which requires no co-factors) to 5α-ergosta-7-en-3β-ol. In a cell-free enzyme assay for maize sterol Δ^{14}-reductase, it has been demonstrated that the Δ^8 product formed is further metabolised to a Δ^7 sterol [19].

This study confirms that both fenpropimorph and fenpropidin are inhibitors of the fungal sterol Δ^{14}-reductase enzyme. The finding that fenpropidin is a more potent inhibitor than fenpropimorph is consistent with that of Baloch and Mercer [11] who compared the efficiency of fenpropimorph, fenpropidin and the structurally related fungicide tridemorph, as inhibitors of both sterol Δ^{14}-reductase and $\Delta^8 \rightarrow \Delta^7$-isomerase fungal enzymes.

Although 3 and 5 show a better enzyme inhibitory activity than either the standard compounds 1 and 2, when tested on wheat against *Erysiphe graminis* (160 mg of active ingredient per litre), 3, 4 and 5 offered no protection [20]. The weak activity of compound 4 in the enzyme assay could be explained by the close proximity of the benzyl group to the N atom which may result in steric hindrance preventing the nitrogen from binding to the active site of the enzyme.

Taton *et al.* [19], who tested a series of six decaline compounds (including the three N-substituted 8-azadecalines used in this study), found molecules of this class to be inhibitors of sterol Δ^{14}-reductase, sterol $\Delta^8 \rightarrow \Delta^7$-isomerase and cycloeucalenol-obtusifoliol isomerase in maize [21]. It is known that fenpropimorph and fenpropidin additionally inhibit fungal sterol $\Delta^8 \rightarrow \Delta^7$-isomerase [11] and also possibly squalene epoxidase in the case of fenpropimorph when tested against certain fungi [22]. The N-substituted 8-azadecalines studied in this work have also been found to inhibit fungal sterol $\Delta^8 \rightarrow \Delta^7$-isomerase [23]. Such a dual mode of action can be explained on the assumption that both isomerase and reductase enzymes proceed by the formation of an HEI at carbons 8 and 14 on the sterol molecule [6]. Because of the close proximity of these two carbon atoms, inhibitors containing a nitrogen atom with a delocalized charge capable of mimicking both C-8 and C-14 carbocationic HEIs are therefore possible. An inhibitor having more than one site of action is desirable since such inhibitors are less likely to give rise to fungicide resistance in the field [11].

A cell-free enzyme assay has been developed for inhibitors of fungal sterol Δ^{14}-reductase using a radiolabelled sterol substrate and radio-detection HPLC. With this procedure it is possible to use a crude enzyme preparation from yeast without the problem of background sterols contaminating the HPLC pattern. This method can be used for the routine screening of candidate inhibitors of fungal sterol Δ^{14}-reductase.

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