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Influence of New Fenpropimorph Fungicides on the Growth and Sterol Composition in *Saccharomyces cerevisiae*: Relationship Between Structure and Activity

JAN ŠAJBIDOR, MARTIN LAMAČKA, ŠTEFAN BALÁŽ*, LAI MAI HUONG, AND ZUZANA CIESAROVÁ

*Department of Biochemical Technology, Chemical Faculty, Slovak Technology University, Radlinského 9, 812 37 Bratislava, Slovak Republic and *Department of Pharmaceutical Sciences, College of Pharmacy, North Dakota State University, Sudro Hall 108, Fargo, ND-58105, USA*

Abstract

The toxicity of fenpropimorph and seven newly synthesized analogues against *Saccharomyces cerevisiae* has been determined in liquid media.

The inhibitory effect of the most efficient derivative is 120 times more than that of standard fenpropimorph. The non-linear relationship between hydrophobicity and toxicity indicates that the binding of the compounds to the receptors does not differ and so the differences in toxicity reflect changes in the rate of metabolism. The presence of inhibitors in the fermentation medium resulted in a reduction in harvested biomass and lipid yield, and changes in sterol composition – the amount of ergosterol decreased whereas the amounts of lanosterol, dihydroergosterol and squalene increased.

The toxicity of the compounds was most influenced by their lipophilicity. Use of this information could lead to development of more potent ergosterol inhibitors.

Fenpropimorph {*N*-[3-(*p*-*t*-butylphenyl)-2-methylpropyl]-*cis*-2,6-dimethylmorpholine} belongs to a new class of the 3-phenylpropylamines with fungal toxicity attributed to inhibition of sterol biosynthesis. Marcireau et al (1990) reported that low concentrations of fenpropimorph inhibited Δ^8-Δ^7-isomerase and higher levels affected Δ^{14}-reductase in *Saccharomyces cerevisiae*. Lorenz & Parks (1991) analysed the sterol profile in fenpropimorph-resistant mutants of *S. cerevisiae* and confirmed the presence $\Delta^{8,14}$ sterols. It seems that the depletion of ergosterol can be correlated with growth inhibition rather than with the presence of Δ^8-sterols (Kelly et al 1994). Ladeveze et al (1993) suggested that unsaturated sterols can efficiently replace ergosterol as bulk membrane component and that the fungistatic effect of fenpropimorph is linked with ergosterol inhibition rather than with the accumulation of abnormal sterols in treated cells. This leads to the arrest of cell proliferation in the unbudded G1 phase of the cell cycle (Marcireau et al 1990). Another possible mechanism of the toxic effect on *S. cerevisiae* was reported by Crowley et al (1994) who detected a significant reduction in uracil and cytosin uptake in the presence of fenpropimorph.

Correspondence: J. Šajbidor, Department of Biochemical Technology, Chemical Faculty, Slovak Technology University, Radlinského 9, 812 37 Bratislava, Slovak Republic.

New, more potent, fungicides could possibly be found by modification of the structure of fenpropimorph. An important tool in the search for new bioactive compounds is the application of quantitative structure–activity relationships (QSAR); such relationships might enable understanding of the mechanism of drug action and also enable forecasting of the activity of a substance of known structure without the need to synthesize it. According to Hansch et al (1963) the biological activity of a substance is a function of its hydrophobic, electronic and steric properties:

$$\log(1/c) = k_1 \log P - k_2 (\log P)^2 + k_3\sigma + k_4 E_s + k_5 \quad (1)$$

where P is the partition coefficient of the substance between 1-octanol and water, σ and E_s are electronic and steric constants, c is the molar concentration of a derivative in a family of related compounds inducing an equivalent biological response, e.g. ID50, and k_1–k_5 are regression

coefficients (Hansch et al 1963). Occasionally electron and steric parameters and substance transport through the biological system can be neglected (in-vitro tests) and equation 1 can be simplified such that the lipophilic parameter (log P) is the measure of hydrophobic interaction between the substance and the receptor:

$$\log(1/c) = k_1 \log P + k \qquad (2)$$

Another physicochemical parameter used in QSAR studies is the molar refractivity (MR), defined by the Lorenz-Lorenz equation:

$$MR = [(n^2 - 1)/(n^2 + 2)]MW/d \qquad (3)$$

where n is refractive index, MW the molecular weight and d the density of the substance. MR is an additive property of organic compounds (Hansch & Calef 1976) and extensive tables of its values for different substituents have been compiled (Hansch et al 1995). The term MW/d in the Lorenz–Lorenz equation is the molar volume of the substance and it is therefore possible to use MR as an approximate criterion of steric influence. The relationship between MR and the coefficient of electric polarizability can be expressed by the equation:

$$MR = (4\pi\alpha N_A)/3 \qquad (4)$$

where α is coefficient of electric polarizability and N_A is the Avogadro constant. It is evident that electric forces also contribute to the MR value (Seydel & Schaper 1979).

In this study we tested the influence of seven newly synthesized derivatives of fenpropimorph on the growth, lipid content and sterol profile of *S. cerevisiae*. We have also developed quantitative structure–activity relationships for these substances.

Materials and Methods

Saccharomyces cerevisiae (strain LH02/2 obtained from B. Janderová, Department of Genetics, Microbiology and Biophysics, Charles University, Prague) was maintained on malt-extract agar at 4°C. Starter cultures of *S. cerevisiae* were incubated in 500-mL flasks, containing 100 mL nutrient medium, on a rotary shaker (28°C, 180 rev min^{-1}, 24 h) to produce approximately 1 mg dry weight of cells (mL medium)$^{-1}$. The nutrient medium contained (g L^{-1}): glucose, 20; yeast autolysate, 3; $(NH_4)_2SO_4$, 5; KH_2PO_4, 2; $MgSO_4.7H_2O$, 1; $CaCl_2$, 0·1; NaCl, 0·1; Na_2HPO_4, 1. Final pH was adjusted to 5·8. The fenpropimorph standard was obtained

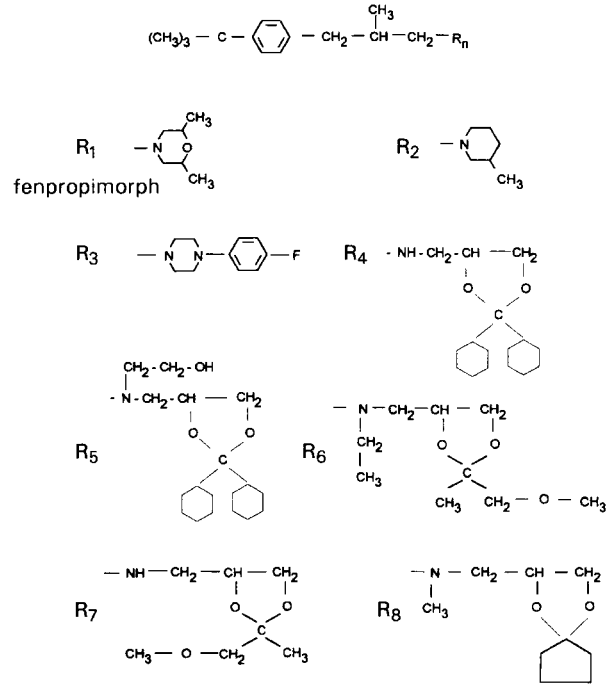

Figure 1. The chemical structures of fenpropimorph (R_1) and its seven derivatives R_2–R_8.

from Sigma (USA). The other derivatives used in our experiments (Figure 1) were synthesized according to Veverka et al (1990).

The antifungal activity of each compound was evaluated by measurement of inhibition of the growth of *S. cerevisiae*. For this purpose, test tubes (10 mL) were filled with culture medium (4·5 mL) with the same composition as that described above but supplemented with 20 g L^{-1} glucose, and various amounts of each derivative (100 to 5000 μM) were added. The antifungal agents were added in ethanolic solution immediately before inoculation with 0·5 mL starter culture. The amount of ethanol never exceeded 1%. Incubation was performed on a rotary shaker (180 rev min^{-1}, 28°C, 31 h). Yeast growth in the presence (treated cultures) or absence (controls) of inhibitors was determined by measuring the optical density of the medium every 2 h. For each compound, the concentration causing a 50% reduction in the growth rate (ID50) was determined using the dose–response curves obtained with at least five experimental concentrations.

For lipid analysis the yeast biomass was extracted by the method of Folch et al (1957) and sterols were analysed by high-performance liquid chromatography (Arnezeder et al 1989).

For formulation of QSAR for *S. cerevisiae* growth inhibition, the properties of the tested compounds that were estimated were the partition

Table 1. Toxicity (ID50) and other properties of fenpropimorph and its seven derivatives.

Compound	Molecular weight	Toxicity*	Log(1/ID50)	Log(partition coefficient)†	Molar refractivity‡	Surface area (Å^2)	Volume (Å^3)
Fenpropimorph	303·5	8870	2·05	5·87	95·0	561	997
R_2	287·5	7480	2·13	6·62	93·7	550	977
R_3	368·5	893	3·05	7·68	114	611	1119
R_4	455·7	49·1	4·31	9·32	138	721	1379
R_5	499·8	185	3·73	9·27	150	720	1433
R_6	377·6	9230	2·04	5·63	112	624	1170
R_7	349·5	16100	1·79	4·68	102	577	1076
R_8	359·6	9440	2·03	5·80	109	599	1105

*ID50 (M). †Calculated by use of ClogP. ‡Calculated by use of Hyperchem.

coefficient (P) of the neutral substance in the system 1-octanol and water, calculated using the ClogP software (BioByte 1995); molar refractivity (MR); surface area; and volume. Molar refractivity, surface area and volume were obtained by use of Hyperchem software (Hypercube 1996) for the molecules minimized by the MM+ force field.

Results and Discussion

Growth of *S. cerevisiae* was inhibited by all the fenpropimorph derivatives tested (Table 1). Toxicity, expressed as ID50, decreased in the order: $R_4 > R_5 > R_3 > R_2 > R_1$ (fenpropimorph) $> R_6 > R_8 > R_7$. The ID50 of the most effective substance (R_4) was approximately 120 times lower than that of fenpropimorph (R_1).

Table 2 summarizes the influence of inhibitor R_4 on the growth, lipid content and the composition of unsaponifiable compounds of *S. cerevisiae* cultivated under aerobic conditions with glucose as the sole carbon source. The presence of inhibitor resulted in a reduction in lipid yield and changes in sterol composition, in addition to a reduction in harvested biomass. The relative percentage of ergosterol decreased and the levels of lanosterol, dihydroergosterol and squalene increased. These observations are in accordance with previous observations of higher ergosterol precursor content in the presence of fenpropimorph (Kelly et al 1994; Ladeveze et al 1993).

The lipophilicity of compounds determines their transport through and accumulation in biological membranes and their non-specific binding to proteins. This property is usually characterized as the partition coefficient, P, between 1-octanol and water. The values of log P for the compounds, estimated by the ClogP method, are summarized in Table 1. The inhibitors differ widely in lipophilicity, the P values covering almost five orders of magnitude.

Molecular size has an important influence on the ability to bind to specific sites on macromolecules. This attribute of the inhibitor molecules was characterized by molar refractivity MR, surface area, and molecular volume (Table 1), properties which are closely related. The correlation coefficients for the dependence of molar refractivity on the surface area and the volume are $r = 0{\cdot}983$ and $r = 0{\cdot}990$, respectively. The surface area and volume are also collinear ($r = 0{\cdot}993$).

Lipophilicity comprises two energetic contributions, formation of a cavity for the molecule and interaction of the molecule with the surrounding solvent. For a homologous series of compounds the interaction of the molecule can be invariant and lipophilicity is dominated by the bulkiness of the molecules. In this circumstance lipophilicity often depends on parameters related to the size of the molecule. For the compounds studied, however, these correlations are marginal (correlation coefficients $r = 0{\cdot}837$ for MR, $r = 0{\cdot}830$ for surface area

Table 2. The influence of three concentrations of compound R_4 on the growth and lipid content of *S. cerevisiae*.

Inhibitor concentration (μM)	Biomass (g L^{-1})	Lipid (mg L^{-1})	Unsaponifiable compounds (%w/w of total content)				
			Ergosterol	Ergosta-5,7,24(28)-tetraenol	Dihydroergosterol	Lanosterol	Squalene
0	1·11	39·8	72·6	13·8	0·8	1·2	0·8
50	0·586	34·0	65·6	8·5	0·7	1·5	–
100	0·361	26·0	53·0	8·8	1·8	4·9	15·2

and r = 0·791 for volume). From the standpoint of QSAR the lack of collinearity between lipophilicity and molecular size of the compounds studied is important, because it enables separate evaluation of the influence of the two properties on toxicity.

Lipophilicity, as the determinant of transport and non-specific non-covalent binding to proteins is the most frequently encountered property in QSAR. The dependence of the toxicity of fenpropimorph derivatives on lipophilicity can be expressed as:

$$\log (1/ID50) = -0{\cdot}96\ (\pm 0{\cdot}41) + 0{\cdot}53\ (\pm 0{\cdot}06) \log P$$
$$(n = 8,\ r = -0{\cdot}966,\ s = 0{\cdot}264) \qquad (5)$$

in which n represents the number of data points, r the correlation coefficient, s the standard deviation from the regression and the numbers in brackets are the confidence limits ($\alpha = 0{\cdot}05$) of the regression coefficients. As can be seen from Figure 2, the less lipophilic compounds deviate from the linear dependence. This deviation can be taken into account by more detailed description of the relationship between toxicity and lipophilicity:

$$\log(1/ID50) = \log [4{\cdot}40 \times 10^{-5}(\pm 2{\cdot}01 \times 10^{-5}) \times P^{0{\cdot}71\ (\pm 0{\cdot}11)} + 1] + 1{\cdot}8\ (\pm 0{\cdot}19)$$
$$(n = 8,\ r = 0{\cdot}983,\ s = 0{\cdot}183) \qquad (6)$$

Published non-linear dependencies of biological activity on lipophilicity usually have a maximum (Hansch & Clayton 1973). The shape depicted in Figure 2 (a curve with a minimum) is observed when the compounds tested do not differ in receptor binding (Balaz et al 1992). The lower activity of the less lipophilic derivatives is because of their faster metabolism compared with lipophilic compounds.

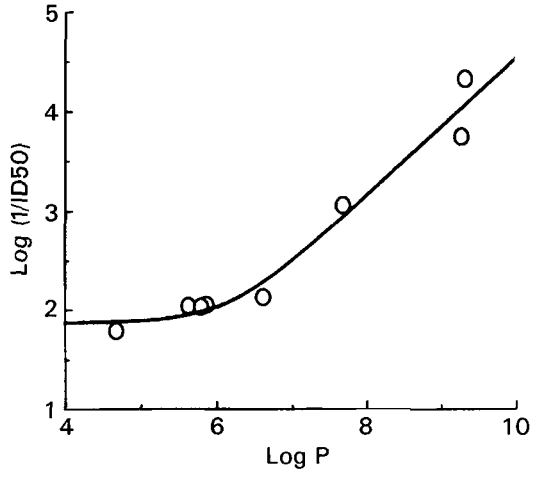

Figure 2. Relationship between the lipophilicity (log P) and toxicity (log ID50) of fenpropimorph derivatives tested on *Saccharomyces cerevisiae* grown under aerobic conditions.

The values of parameters related to the bulkiness of the molecules studied (molar refractivity, surface area and volume) are listed in Table 1. Generally, toxicity (log(1/ID50)) increases with bulkiness but the dependencies are not as smooth, as can be seen in Figure 2 and experimental points are more scattered. The linear correlations are much less significant than for lipophilicity: the correlation coefficients are r = 0·872 for molar refractivity, r = 0·893 for surface area and r = 0·851 for volume.

The comparatively smooth dependence of toxicity on lipophilicity (Figure 2) accounts for more than 96% of the variance of the dependent variable, as indicated by the squared correlation coefficient ($r^2 = 0{\cdot}966$) of equation 6 and so the influence of the other properties of the tested compounds on their toxicity was not investigated. Moreover, equation 6 has a mechanistic basis (Balaz et al 1992). This kind of dependency results from the decrease in the rate of metabolism for lipophilic compounds because their greater accumulation in membranes and their greater protein binding leads to lower concentrations in aqueous phases. The toxicity of the less lipophilic compounds could be increased by introduction of structural features conveying higher metabolic stability. This could lead to development of more potent ergosterol inhibitors with the prospect of medical application to animals and man.

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References

Arnezeder, Ch., Koliander, W., Hampel, W. A. (1989) Rapid determination of ergosterol in yeast cells. Anal. Chim. Acta 225: 129–136

Balaz, S., Wiese, M., Seydel, J. K. (1992) A time hierarchy-based model for kinetics of drug disposition and its use in quantitative structure-activity relationships. J. Pharm. Sci. 81: 849–857

BioByte (1995) ClogP for Windows, version 1.0.0

Crowley, J. H., Lorenz, R. T., Parks, L. W. (1994) Fenpropimorph affects uptake of uracil and cytosine in *Saccharomyces cerevisiae*. Antimicrob. Agents Chemother. 38: 1004–1007

Folch, J., Lees, M., Sloane-Stanley, G. H. (1957) A simple method for the isolation and purification of total lipids from animal tissues. J. Biol. Chem. 226: 497–509

Hansch, C., Calef, D. F. (1976) Structure-activity relationships in papain-ligand interactions. J. Org. Chem. 41: 1240–1243

Hansch, C., Clayton, J. M. (1973) Lipophilic character and biological activity of drugs II: the parabolic case. J. Pharm. Sci. 62: 1–21

Hansch, C., Muir, R. M., Fujita, T., Maloney, P. P., Geiger, C. F., Streich, M. J. (1963) The correlation of biological activity of plant-growth regulators and chloromycetin derivatives with Hammett constants and partition coefficients. J. Am. Chem. Soc. 85: 2817–2824

Hansch, C., Leo, A., Hoekman, D. (1995) Exploring QSAR-Hydrophobic, Electronic, and Steric Constants. ACS Professional Reference Book, Washington, DC

Hypercube (1996) Hyperchem for Windows, version 4.5

Kelly, D. E., Rose, M. E., Kelly, S. L. (1994) Investigation of the role of sterol delta 8-7 isomerase in the sensitivity of *Saccharomyces cerevisiae* to fenpropimorph. FEMS Microbiol. Lett. 122: 223–226

Ladeveze, V., Marcireau, C., Delourme, D., Karst, F. (1993) General resistance to sterol biosynthesis inhibitors in *Saccharomyces cerevisiae*. Lipids 28: 907–912

Lorenz, R. T., Parks, L. W. (1991) Physiological effects of fenpropimorph on wild type *Saccharomyces cerevisiae* and fenpropimorph-resistant mutants. Antimicrob. Agents Chemother. 35: 1532–1537

Marcireau, C., Guilloton, M., Karst, F. (1990) In vivo effects of fenpropimorph on the yeast *Saccharomyces cerevisiae* and determination of the molecular basis of the antifungal property. Antimicrob. Agents Chemother. 34: 989–993

Seydel, J. K., Schaper, K. J. (1979) Chemical Structure and Biological Activity of Substances. Methods of Quantitative Structure-Activity Analysis. Verlag Chemie, Weinheim

Veverka, M., Štibrányi, L., Szemes, F. (1990) The method for the preparation of phenylpropylamine derivatives. Czechoslovak Pat. Appl. 11790