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INHIBITION OF MICROBIAL CHOLESTEROL OXIDASES BY DIMETHYLMORPHOLINES

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Summary—Cholesterol oxidase is a potentially important enzyme in steroid transformations, catalysing the conversion of 3-hydroxy-5-ene steroids to 3-keto-4-ene derivatives via a 3-keto-5-ene intermediate. Morpholine derivatives, especially fenpropimorph and tridemorph, were found to block selectively the isomerisation activity of cholesterol oxidases isolated from *Nocardia erythropolis*, *Streptomyces* sp., *Pseudomonas testosteroni* and *Schizophyllum commune*. These enzymes differ strongly in physical characteristics and catalytic behaviour. The effectiveness of the inhibitors varied with the cholesterol oxidase tested. Fenpropimorph was most effective with each of the 4 enzymes, 50 mg/l inhibiting about 50% of the enzyme activity. Inhibition was instantaneous and followed a reversible competitive mechanism in *Streptomyces* sp. and a reversible non-competitive mechanism in *Nocardia erythropolis* and *Schizophyllum commune*. An irreversible type of inhibition was observed for *P. testosteroni* cholesterol oxidase.

INTRODUCTION

A substantial portion of the vast steroid drug production is accomplished by microbial or enzymatic transformations [1, 2]. The optimisation and development of novel enzymatic and microbial reactions is therefore of considerable interest. A potentially important enzyme in steroid conversions is cholesterol oxidase, which is at present mostly used in serum cholesterol analysis and steroid identification [3, 4]. Cholesterol oxidase catalyses the conversion of 3-hydroxy-5-ene steroids to their 3-keto-4-ene derivatives via a 3-keto-5-ene intermediate (Fig. 1). Separation of these two activities could be useful for specific steroid transformations. One approach to accomplish this is the development of selective inhibitors for one of these enzyme activities.

In this paper we describe several inexpensive inhibitors for this purpose such as fenpropimorph, tridemorph and fenpropidin. These sterol biosynthesis inhibiting fungicides for agronomic purposes were found to be effective inhibitors of the cholesterol oxidases of *Nocardia erythropolis*, *Streptomyces* sp., *Pseudomonas testosteroni* and *Schizophyllum commune in vitro*. Effectiveness and inhibition mechanism varied with each of the enzyme-inhibitor combinations tested, although the isomerisation was always the target of inhibition.

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EXPERIMENTAL

Cholesterol oxidases

Cholesterol oxidase from *Nocardia erythropolis* was obtained from Boehringer (Mannheim, West Germany) as a 3 M NaCl solution. The enzyme was desalted with a Sephadex G 25 PD 10 column (Pharmacia, Uppsala, Sweden) with 50 mM potassium phosphate, pH = 7.0, as elution buffer. Sodium chloride was adequately removed and the enzyme was diluted 5-fold by this treatment. This enzyme solution was kept on ice and was used during one day. No loss of activity could be detected.

Cholesterol oxidase from *Schizophyllum commune* and from *Streptomyces* sp. were purchased from Sigma (St. Louis, Mo., U.S.A.) as lyophilized powders with reported activities of 15.6 and 25 U/mg, respectively.

Cholesterol oxidase from *Pseudomonas testosteroni* was purchased from Sigma (St. Louis, Mo., U.S.A.) as a lyophilized powder containing 3.3 U/mg of β-activity. The oxidase assay with this enzyme contained a 50-fold excess of NAD when compared to the molar amount of steroid substrates.

Enzyme assays

Enzyme assays were carried out in 1 ml of total volume under the optimized conditions for each enzyme, as described in Table 1. The formation of 3-keto-4-ene steroids was measured with an LKB Biochrom Ultraspec 4050 UV-VIS spectrophotometer in 1 cm quartz cuvettes at 239 nm. The

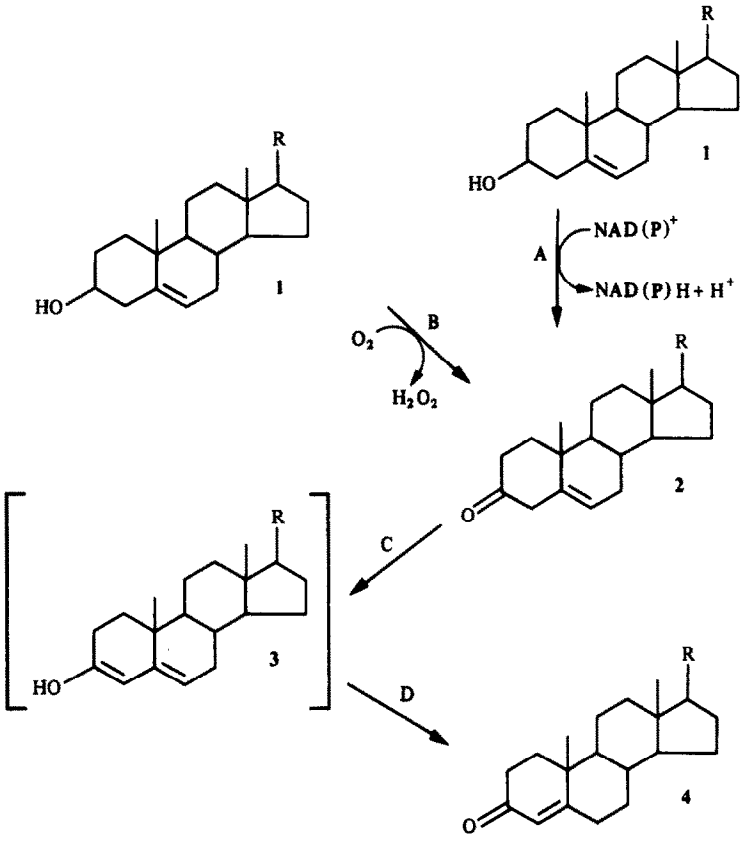

Fig. 1. The oxidation (A or B) and isomerisation (C, D) reactions catalysed by cholesterol oxidase. Names: 1 = 3-hydroxy-5-ene steroid, 2 = 3-keto-5-ene steroid and 4 = 3-keto-4-ene steroid.

constituents of the assay solution were mixed before cholesterol oxidase was added to start the reaction. To follow the reaction, the spectrophotometer was coupled to a printer which was programmed to print out the absorption value every 5 s. The molar extinction coefficients used for cholest-4-en-3-one and progesterone were 17,340 and 18,506 l/mol·cm, respectively [5]. NAD reduction was followed at 340 nm. A value of 6230 l/mol·cm was used as the NADH molar extinction coefficient [6].

Determination of Michaelis–Menten constants

Using the optimised assays for each enzyme, the Michaelis–Menten constants V_{max} and K_m were calculated by measuring the initial reaction velocities at a fixed inhibitor concentration and at 8–10 different substrate concentrations. Reciprocal Lineweaver–Burke plots were constructed and V_{max} and K_m were determined. These values were independently checked using the method of Cornish-Bowden and Eisenthal [7, 8]. Results from both methods agreed very well.

Steroid analyses

Capillary gaschromatography (GC) and thin-layer chromatography (TLC) were carried out as described before [5, 9]. For GC, a Packard 436 gaschromatograph was used. Steroids were analysed as their MO-TMS-derivatives on a 25 m CP Sil 5 CB fused silica capillary column, 0.22 mm i.d. (Chrompack, Middelburg, The Netherlands).

Chemicals

All steroids and NAD were purchased from Sigma (St. Louis, Mo., U.S.A.). Steroids were checked for purity by thin layer chromatography (TLC) and capillary gaschromatography (GC). Inhibitors were provided by TNO (Institute of Applied Chemistry,

Table 1. Optimal assay conditions for the transformation of 3-hydroxy-5-ene steroids to their 3-keto-4-ene derivatives by four different cholesterol oxidases

	Optimum conditions					
Enzyme source	pH	Temperature (°C)	Buffer (mM)	Detergent (mg/l)	Isopropanol (% v/v)	Substrate
N. erythropolis	7.00	30	50 P	2.5	25–30	Cholesterol
Streptomyces sp.	7.50	25	100 P	2.0	10, 25	Cholesterol
P. testosteroni	8.85	25	100 T	2.5	12.5	Pregnenolone
S. commune	5.15	37	100/7 A/P	0	2, 20	Cholesterol

Abbreviations: A = sodium acetate, P = sodium phosphate, T = Tris–HCl. Triton-X 100 was used as a detergent.

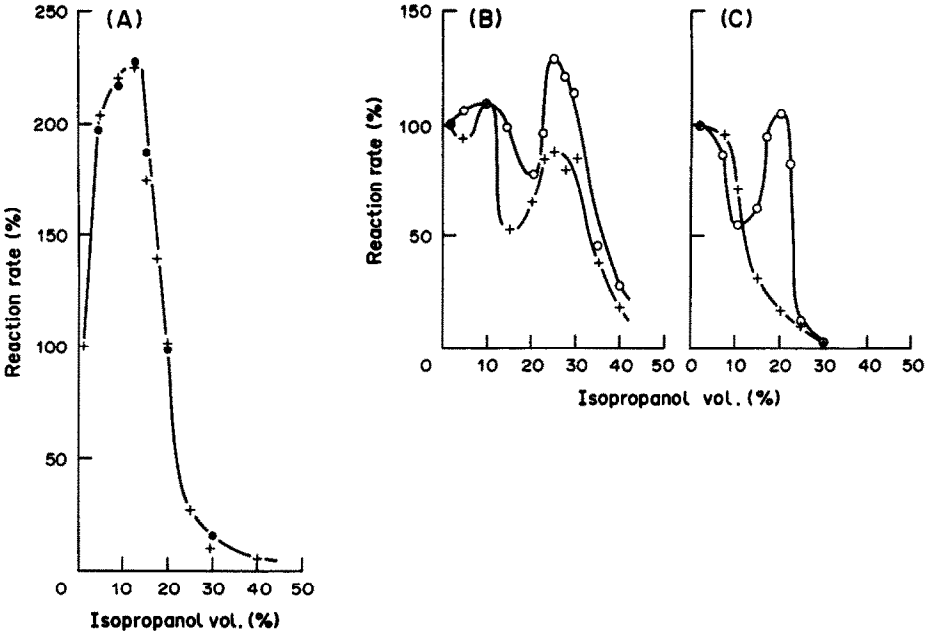

Fig. 2. Effects of isopropanol on the isomerase (○), the oxidase (●) and the total (+) activities of the cholesterol oxidases from *P. testosteroni* (A), *Streptomyces* sp. (B) and *S. commune* (C) (assay conditions in Table 1).

Zeist, The Netherlands) and were chemically pure racemates, unless stated otherwise. All other reagents were of analytical grade.

RESULTS

Enzyme assay development

As all substrates, products and inhibitors are poorly soluble in aqueous media, we had to develop assay solvents with apolar characteristics, compatible with the enzymes used. We examined aqueous isopropanol/buffer mixtures as not all cholesterol oxidases are compatible with water saturated organic solvents [5]. Optimum pH, temperature, detergent concentration and isopropanol content were determined for each of the 4 cholesterol oxidases used (Table 1). The best substrate for each enzyme was also identified out of a group of steroids consisting of sitosterol, stigmasterol, cholesterol, pregnenolone and 3-hydroxy androst-5-en-17-one.

We have previously reported that isopropanol has significantly different effects on the isomerase versus the oxidase activities of *N. erythropolis* cholesterol oxidase [5]. Therefore, the effects of isopropanol on these activities were measured separately for each of the enzymes, using cholesterol and cholest-5-en-3-one (5-cholestenone) as substrates for the conversion to cholest-4-en-3-one (4-cholestenone). As we did not have pregn-5-en-3,17-dione, which is the appropriate substrate for measurement of the separate isomerisation reaction of *P. testosteroni* cholesterol oxidase, only the total cholesterol oxidase reaction was investigated, using prenenolone as the substrate for the conversion to progesterone (Fig. 2).

Non-enzymatic isomerisation

The isomerisation of a 3-keto-5-ene steroid to a 3-keto-4-ene steroid is an energetically favourable process as a conjugated enone is formed. Because the activation energy barrier is relatively low, acid or base catalysed non-enzymatic isomerisation might occur. Therefore, the influence of the pH on the non-enzymatic conversion of 5-cholestenone to 4-cholestenone in 100 mM buffers containing 15 and 30% (v/v) of isopropanol was determined. Figure 3 shows a slow increase in non-enzymatic isomerisation

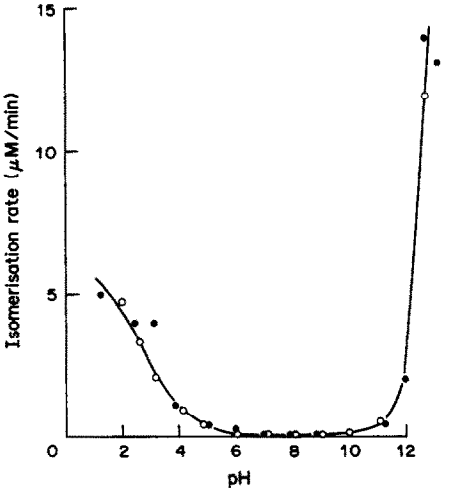

Fig. 3. The non-enzymatic conversion of 5-cholestenone to 4-cholestenone as a function of pH. Measurements were carried out at 30°C in 100 mM HCl, sodium acetate, sodium phosphate or Tris–HCl buffer containing 15 and 30% (v/v) isopropanol. The initial substrate concentration was 52.0 μM.

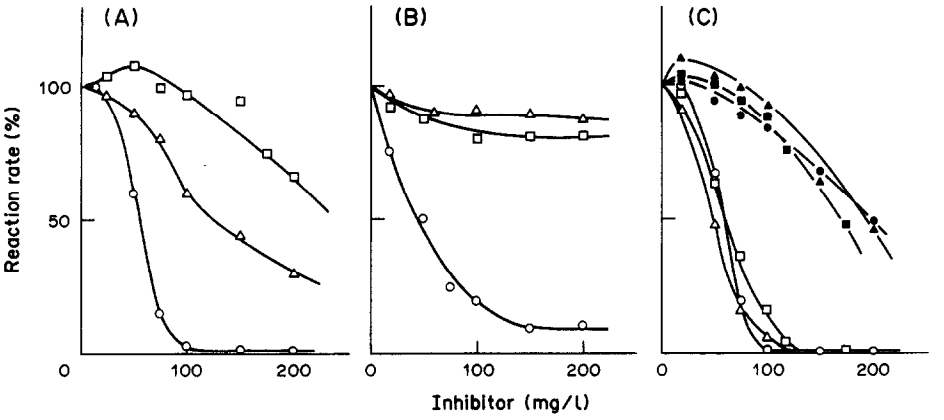

Fig. 4. Effect of dimethylmorpholines on cholesterol oxidase activities *in vitro*. A,B = inhibition of the isomerisation of 5-cholestenone to 4-cholestenone by *Streptomyces* sp. and *S. commune* cholesterol oxidase; C = inhibition of the oxidation (solid symbols) or total reaction (open symbols) of pregnenolone to progesterone by *P. testosteroni* cholesterol oxidase. Symbols: ○ = fenpropimorph, △ = tridemorph, □ = fenpropidin.

rates at pH < 6 and a steep increase at pH > 11. This implied that some care was necessary in the assay with *S. commune* cholesterol oxidase only, its optimum pH being about 5.15. Therefore, all values obtained for the isomerisation reaction using this enzyme, have been corrected for the non-enzymatic reaction. The amount of isopropanol had no additional effect on the non-enzymatic isomerisation. We also investigated the non-enzymatic conversion of cholesterol and pregnenolone to their corresponding 3-keto-4-ene derivatives. No product formation was observed between pH values ranging from 3 to 12 in assays containing 25% isopropanol. This implies that cholesterol oxidase activity is required for the conversion of 3-hydroxy-5-ene steroids to 3-keto-4-ene steroids.

Inhibition of cholesterol oxidases

The dimethylmorpholines fenpropimorph and tridemorph and the morpholine fenpropidin were tested as inhibitors in the optimised cholesterol oxidase assays. Using *N. erythropolis*, *Streptomyces* sp. and *S. commune* cholesterol oxidase, the total and the isomerisation reaction were separately investigated by measurement of the rate of 4-cholestenone production out of cholesterol and 5-cholestenone, respectively. For *P. testosteroni* cholesterol oxidase, both the total and the oxidation reaction rates were investigated by using pregnenolone as the substrate to be converted to progesterone. The oxidation reaction was followed by determining the NADH formation rate at 340 nm. The total reaction rate was measured by detection of progesterone formation at 239 nm. The results are shown in Fig. 4.

No differences were observed between the inhibition of the total and the isomerisation reaction with *N. erythropolis*, *Streptomyces* sp. and *S. commune* cholesterol oxidase by any of the potential inhibitors tested. Therefore, the isomerisation seems to be the target of inhibition. Consequently, clear differences in effectiveness were observed between the inhibition of the total versus the separate oxidation reaction with *P. testosteroni* cholesterol oxidase. The oxidation reaction was far less sensitive to inhibition by either of the inhibitors than the total reaction, again indicating that the isomerisation is the target of inhibition. Fenpropimorph proved to be an effective inhibitor with each of the 4 cholesterol oxidases tested. Tridemorph was equally as effective as fenpropimorph with *N. erythropolis* and *P. testosteroni* cholesterol oxidase, less effective with the *Streptomyces* enzyme and not functional with *S. commune* cholesterol oxidase. Fenpropidin was a very good inhibitor of *P. testosteroni* oxidase but had minor if any effect on the other 3 enzymes.

Inhibition kinetics and mechanism

In order to get some information on the mechanism of inhibition by fenpropimorph, the Michaelis–Menten constants V_{max} and K_m were determined for the isomerisation or oxidation reaction with and without fenpropimorph present. The optimal assay conditions of Table 1 were employed. Table 2 summarises the results and indicates that the isomerase from *Streptomyces* sp. was inhibited competitively, whereas the *N. erythropolis* and *S. commune* isomerases were inhibited non-competitively by fenpropimorph. For *P. testosteroni* no straight curves could be obtained in the Lineweaver–Burke plots, nor did the method of Cornish–Bowden and Eisenthal[7, 8] lead to unambiguous results. Inhibition was found to be instantaneous with each of the cholesterol oxidases tested. This was confirmed by adding inhibitor to a non-inhibited reaction. Reaction rates always decreased immediately.

DISCUSSION

Cholesterol oxidase characteristics

In this study we investigated the inhibition of cholesterol oxidases which are produced by the

Table 2. Michaelis–Menten constants of various cholesterol oxidases

Enzyme source	V_{max} (U/mg)	K_m (μM)	Inhibition by fenpropimorph: Inhibitor (mg/l)	V_{max} (U/mg)	K_m (μM)
N. erythropolis	14	7	20	8	14
Streptomyces sp.	7.0	67	40	7.0	111
P. testosteroni	3.8	27	ND	—	—
S. commune	28.9	140	20	22.9	142

The oxidation reaction was investigated for *P. testosteroni* cholesterol oxidase, the isomerization reaction for the other three enzymes. Fenpropimorph was used as the inhibitor. ND = not determined.

Gram-positive bacteria *N. erythropolis* and *Streptomyces* sp., by the Gram-negative *P. testosteroni* and by the fungus *S. commune*. Some characteristics of these cholesterol oxidases are summarised in Table 3.

Cholesterol oxidases generally have rather broad pH and temperature optima and are able to convert a variety of the usual 3-hydroxy-5-ene steroids to their 3-keto-4-ene derivatives. Cholesterol is normally the substrate of choice. Other steroids to be converted must be flat, implying a *trans* fusion of the A and B rings, and have no other substituents than hydrogen in axial α-positions in these 2 rings. The C-17 side chains may vary in length from 0 to 10 carbon atoms and may have substituents such as hydroxyl groups [10]. Some minor deviations from these 'rules' are the cholesterol oxidases from *S. commune*, able to convert lanosterol, and *P. testosteroni*, which hardly transforms steroids with side chains longer than 2 carbon atoms. These substrate specificities have been extensively reviewed in the literature [11–13]. The requirements mentioned above put severe restrictions on the development of potential inhibitors based on structural similarities with substrates.

Dimethylmorpholines as inhibitors of sterol metabolism

The cholesterol oxidase inhibitors tested in this study belong to the class of dimethylmorpholines and structural analogues thereof. These compounds are known to block several sterol bioconversions in fungi and higher plants, such as C-7 to C-8 isomerisation and C-14 double bond reduction [14]. Dimethylmorpholines inhibit these sterol transformations by the formation of morpholinium ions, high energy intermediates which strongly resemble the sterol carbonium ions in their transition states [15, 16].

In a previous study, we showed that fenpropimorph and tridemorph were among the most effective inhibitors of the isomerase activity of *N. erythropolis* cholesterol oxidase out of a group of structural analogues such as amorolfine, fenpropidin, dimethylfenpropidin, fenpropimorph-*N*-oxide and the optically pure *cis*- and *trans*-methyl isomers of the morpholine ring of fenpropimorph [5]. In this study, we tried to extend the applicability of this class of inhibitors to other cholesterol oxidases.

Assay optimisation

The optimal cholesterol oxidase assay conditions with respect to temperature, pH and substrate were established and are in good agreement with literature [9, 11, 17–20]. Assays for these enzymes require Triton-X 100 and isopropanol to solubilise substrates and inhibitors [5]. The optimal Triton-X 100 concentrations were 2.0–2.5 mg/l for the *N. erythropolis*, *P. testosteroni* and *Streptomyces* sp. enzymes. In contrast, *S. commune* cholesterol oxidase was inactivated by Triton-X 100. Therefore, no detergent was added in assays with this enzyme. The effects of large amounts of isopropanol on cholesterol oxidase were determined separately for the isomerase and oxidase activities. With the exception of *P. testosteroni* cholesterol oxidase, all isomerases displayed a minimum of activity around 10% isopropanol and a maximum at 20–25% (v/v). This effect was most pronounced with *N. erythropolis* cholesterol oxidase, the maximum isomerisation rate being 4.7-fold the rate in the absence of isopropanol [5]. For *Streptomyces* sp. and *S. commune* cholesterol oxidases, isopropanol caused a 1.2-fold increase in isomerase activity. The oxidase activity was stimulated by isopropanol only for the *N. erythropolis* enzyme, leading to a 1.7-fold increase at 22.5% isopropanol, whereas *Streptomyces* sp. cholesterol oxidase showed a second peak of 85% residual activity at 25% isopropanol. The total activity of the *S. commune* enzyme was inhibited by isopropanol addition. These results suggested that the 2 activities of the cholesterol oxidases could be influenced separately. For *P. testosteroni*, isopropanol stimulated the total cholesterol oxidase reaction, resulting in a 2.2-fold increase of enzyme activity at 12.5% isopropanol. When the oxidase activity was measured separately, it displayed the same correlation with isopropanol content. Since the isomerase is not rate limiting, its reaction must have

Table 3. Some characteristics of microbial cholesterol oxidases

Enzyme source	mol. wt (Da)	pH_{opt}	$Temperature_{opt}$ (°C)	Cofactor	Ref.
N. erythropolis	55,000	7.0	32	—	20
Streptomyces sp.	34,000	7.2	45	—	17
P. testosteroni	100,000	8.5	25	NAD(P)	9, 18
S. commune	53,000	4–5	37	FAD-cov.	11, 19

Table 4. Inhibitor concentrations causing 50% inhibition of cholesterol oxidase activity

Enzyme source	Fenpropimorph (mg/l)	Fenpropimorph ratio	Tridemorph (mg/l)	Tridemorph ratio	Fenpropidin (mg/l)	Fenpropidin ratio
N. erythropolis						
Isomerization	35	1580	40	1850	>200	>10,000
Total reaction	40	1815	43	1990	>200	>10,000
Streptomyces sp.						
Isomerization	58	325	133	761	>200	>1245
Total reaction	61	340	ND	—	ND	—
P. testosteroni						
Oxidation	160	2640	200	3370	161	2950
Total reaction	58	955	50	842	62	1135
S. commune						
Isomerization	56	1630	>200	>5950	>200	>6470
Total reaction	57	1660	>200	>5950	>200	>6470

Substrates were 5-cholestenone and cholesterol for the isomerization and total reaction, respectively, in *N. erythropolis*, *Streptomyces* sp. and *S. commune*.

Pregnenolone was the substrate for the two *P. testosteroni* reactions. Ratios give the approximate molar ratio of inhibitor to enzyme at 50% inhibition. The assay conditions are given in Table 1.

been stimulated to the same extent or more [20]. In all assays thus optimised, solubilities of steroids and inhibitors were raised from *ca* 5 mg/l to over 200 mg/l, which was sufficient for our purposes.

Inhibition of microbial cholesterol oxidases

Three representatives of the class of dimethylmorpholine-like compounds were tested in the optimized *in vitro* assays in this study. Fenpropimorph and tridemorph were applied as successful inhibitors of *N. erythropolis* cholesterol oxidase and fenpropidin as a control compound, being without any inhibitory effect with that enzyme [5]. As summarised in Table 4, fenpropimorph was an effective inhibitor with each of the 4 enzymes tested, 50 mg/l causing about 50% inhibition. Tridemorph was effective with *N. erythropolis* and *P. testosteroni* isomerase, whereas the *Streptomyces* sp. enzyme was less sensitive and *S. commune* cholesterol oxidase was hardly inhibited at all. To our surprise, fenpropidin was equal to fenpropimorph and tridemorph in the inhibition of *P. testosteroni* isomerase and slightly effective with the *Streptomyces* sp. enzyme. Hardly any inhibition was found with *N. erythropolis* and *S. commune* cholesterol oxidase by this compound. These results reconfirmed that the inhibitor structure was of great importance.

The mechanism of isomerase inhibition by fenpropimorph was found to be non-competitive with *N. erythropolis* and *S. commune* cholesterol oxidases and competitive using the *Streptomyces* sp. enzyme. In our view, inhibition results from blocking the active site of the enzyme due to strong interactions between enzyme and inhibitor. The degree of structural and electronic resemblance between inhibitor and steroid substrate or product determined the effectiveness of an inhibitor. Thus, we were able to show that for inhibition of *N. erythropolis* cholesterol oxidase the presence of the 2 methyl groups, a long side chain and a tertiary nitrogen atom were of great importance. Obviously, each of the 4 enzymes tested in this study had its own specific requirements with respect to the optimal inhibitor structure. Therefore, it appears possible that a compound binds very strongly to the *N. erythropolis* and *S. commune* enzymes resulting in non-competitive inhibition, whereas the same compound binds more loosely to *Streptomyces* sp. cholesterol oxidase, resulting in a competitive mechanism of inhibition. No straight lines were obtained in the Lineweaver–Burke plots for *P. testosteroni* cholesterol oxidase. Therefore, we expect an irreversible mechanism of inhibition rather than a reversible one for this enzyme.

A few groups of compounds are known as cholesterol oxidase inhibitors. The acetylenic seco-steroids such as 5,10-seco-19-nor-5-cholestyne-3,10-dione proved to be effective inhibitors of *N. erythropolis* and *P. testosteroni* cholesterol oxidases. They irreversibly inhibit both enzymatic reactions by alkylating the active site after their conversion into allenic ketones by the enzyme [21, 22]. Inhibition was found to be time-dependent. Two other classes of inhibitors are known for *P. testosteroni* cholesterol oxidase: the 3-hydroxy-1,3,5-estratrienes and the 3-β- or 17-β-oxiranyl steroids. Inhibition by the estratrienes is most probably based on the high affinity of the oxidase active site for these compounds. About 50% of cholesterol oxidase inhibition was found at a ratio of *ca* 1 inhibitor molecule per 3 substrate molecules [13]. The oxiranes bind irreversibly and covalently to an aspartate-carboxyl residue present in the active site. This binding is time dependent and occurs at the α-face of the steroid molecule, possibly via backward binding [23]. So far, these oxiranes have not been tested with *N. erythropolis* cholesterol oxidase, but the same effects could be expected whenever a carboxyl residue is present in or near the active site.

Concluding remarks

It was demonstrated that dimethylmorpholines, especially fenpropimorph and tridemorph, are able to block selectively the isomerase activity in 4 types of cholesterol oxidases, all differing in producing microorganism, cofactors required, substrate specificities and optimum assay conditions. Although differences in effectiveness and action mechanism were observed

for the various combinations of enzymes and inhibitors studied, (dimethyl-) morpholines may be seen as general cholesterol oxidase inhibitors. The application of this class of compounds as inhibitors of microbial steroid transformations *in vivo* will be the subject of further investigations [24]. In addition, since there is an analogy in the functioning of inhibitors of microbial cholesterol oxidases *in vitro* and their inhibitory activity in sterol biosynthesis in higher plants and fungi *in vivo*, this could be a starting point for the development of a simple and fast fungicide screening system. Such a system could provide an extra tool in the development or the identification of novel fungicides for agronomic purposes.

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