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Enzymatic preparation of optically active fungicide intermediates in aqueous and in organic media

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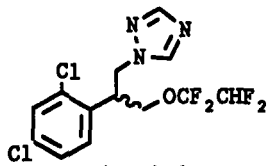
Abstract- Pure stereoisomers of two new triazole and morpholine fungicides were prepared starting from enzymatically synthesized chiral alcohols intermediates. Two resolution strategies were compared: lipase-catalyzed hydrolysis of corresponding acetates in water and lipase-catalyzed transesterification of alcohols in organic solvent. The antifungal activity of optically pure enantiomers of the synthesized fungicides were investigated *in vitro* and *in vivo* against a variety of fungi, showing an activity ratio (R-form / S-form) up to 400.

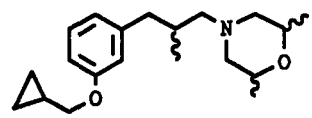
INTRODUCTION

The study of bio-active molecules has shown that for compounds with a chiral character the enantiomers, and in general sterical isomers, can dramatically differ in their capacity to interact with a certain site of action, the "receptor site" on the specific receptor molecule or the "active site" on a particular enzyme (ref. 1).

It is therefore not surprising that also among triazole and morpholine fungicides, which inhibit ergosterol biosynthesis, stereoisomerism plays a very important role (ref. 2). It may determine fungitoxicity, effect on plant physiology, such as plant-growth regulation (PGR) activity, metabolic fate in fungi and, possibly, mode of action and behavior inside the plant. The mode of action of the various stereoisomers can be identical,though quantitatively different, or dissimilar. In the latter case, the "inactive" isomer(s) can be indifferent or even antagonistic, or they can display side-effects, which are beneficial or even unwanted. Consequently we had interest in preparing pure stereoisomers of two new fungicides 1 and 2, to compare their biological activity.



(R,S)-1



CIS-TRANS (R,S)-2

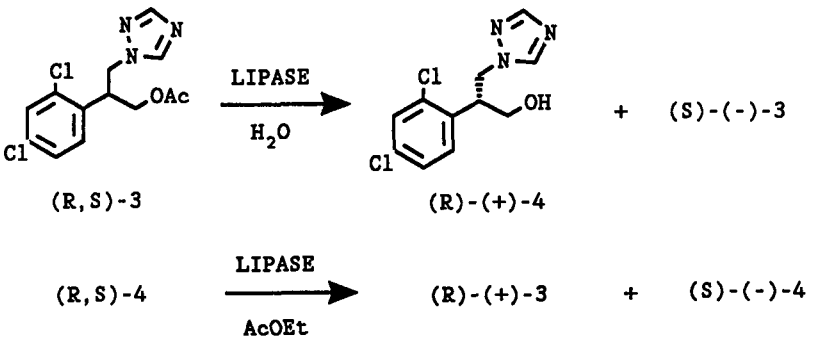
Techniques for stereospecific synthesis and separation of isomers, including enantiomers, are rapidly evolving. Synthesis based on stereoselective catalysts, including enzymatic conversions, play an important role. It is becoming more and more apparent that biotransformations can offer significant advantages over classical chemical methods in the synthesis of physiologically active compounds (ref. 3). Next to the chemo- and regiospecificity often associated with biocatalysis, the observed diastereo- and enantioselectivity of

enzyme-catalyzed reactions make them especially attractive for the preparation of chiral building blocks in the production of pharmaceutical (ref.4) and agrochemicals (ref. 5). The most versatile class of biocatalysts is the hydrolases group comprising such enzymes as proteases, esterases and lipases. These non-cofactor requiring enzymes accept a wide variety of structurally different compounds as substrates, showing a high degree of stereoselectivity. In addition they can be used in almost anhydrous organic solvents, were they can catalyze reactions that cannot be conducted in aqueous solvent (ref. 6). In this paper we report the synthesis of optically active alcohols 4,6 and 7 by lipase catalyzed hydrolysis of the corresponding esters or by lipase catalyzed transesterification of alcohols and their use as chiral starting material in the preparation of optically pure fungicides 1 and 2. We also report the preliminary results of tests performed to investigate *in vivo* and *in vitro* fungitoxicity of the pure stereoisomers.

PREPARATION OF CHIRAL ALCOHOLS

Alcohol 4 was chosen as optically active precursor of triazole fungicide 1. Several commercially available lipases were tested both in the hydrolysis of the corresponding racemic ester 3 and in the transesterification of alcohol 4 (Scheme 1).

Scheme 1



Enzymatic hydrolysis were carried out in phosphate buffer pH 7 at 35°C. The pH was kept constant by addition of 0.1 M NaOH. Transesterification reaction were carried out by adding the powdered enzymes to a solution of 4 in ethyl acetate, which acted both as acylating agent and reaction medium.

The optical purity of 4 was determined by GLC of the corresponding MTPA (Mosher acid) ester (ref. 7). As shown in table 1, Porcine pancreatic lipase (PPL) was more stereoselective than lipases from

TABLE 1

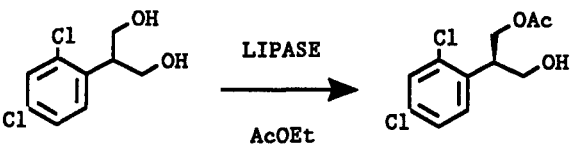
SUBSTRATE	ENZYME	SOLVENT	CONV.%	ESTER 3		ALCOHOL 4	
				e.e.%	CONF.	e.e.%	CONF.
3	PL [a]	H_2O [c]	75	22	R	6	S
3	CCL[b]	H_2O	30	9	R	19	S
3	PPL	H_2O	50	≥95	S	≥95	R
4	PL	AcOEt[d]	70	7	S	18	R
4	PPL	AcOEt	50	≥95	R	≥95	S

a) *Pseudomonas sp.* lipase. b) *Candida cylindracea* lipase.
c) Hydrolytic reactions were performed in phosphate pH 7, 0.05 M (200ml) and dioxane (6 ml) at 35°C; substrate, 25 mmol; lipase ,2 g. d) Transesterification reactions were performed in ethyl acetate (200ml) at 35°C; substrate, 25 mmol; lipase 2 g.

microorganisms. No significant change in enzymes stereoselectivity was noticed moving from water to organic solvent. Optically pure 4 was then transformed into 1 by treating with tetrafluoroethylene and potassium hydroxide. Both the enantiomers of 1 were obtained with optical purity ≥ 95%.

A second synthetic pathway to the R enantiomer of 1, which has been proved to be the more active one, was then developed starting from the prochiral precursor 5. We studied both the hydrolysis of the corresponding diacetate and the transesterification of the diol 5 to give the chiral monoacetate 6. The advantage of this approach is that, in principle, all the substrate is utilized while kinetic resolution gives a maximum yield of 50% and the unwanted isomer has to be racemised or discarded.
When 2-(2,4-dichlorophenyl)-1,3-diacetoxypropane was submitted to lipase-catalyzed hydrolysis, we detected the initial formation of the desired monoacetate 6, followed by an extensive hydrolysis of the second ester group producing the diol 5 (approximately 70%). This side-reaction could not be suppressed by any change of experimental conditions. Lipase-catalyzed transesterification of the diol 5 in organic solvent, instead afforded the monoester 6 in high yield and purity (Scheme 2).

Scheme 2



5 (R)-(+)-6

The transesterification reaction was conducted as described previously for the alcohol 4. Several commercially available hydrolytic enzymes were tested but only Porcine pancreatic lipase and lipase from *Pseudomonas sp.* proved to be effective catalysts (Table 2).

TABLE 2

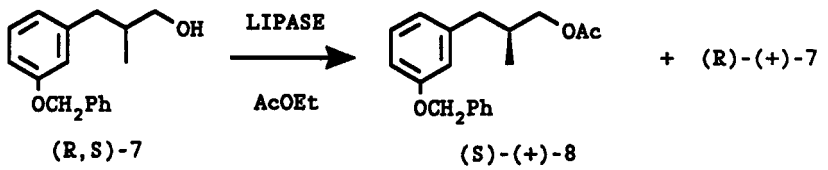
ENZYME [a]	AMOUNT OF ENZYME(mg/ml)	TIME(hr)	CONV.%	ESTER 6 e.e.%	ESTER 6 CONF.
CCL	75	70	80	24	R
PL	4	15	80	81	R
PPL	35	15	70	53	R
PPL\celite [b]	40	8	95	95	R
PPL\celite [c]	40	16	95	99	R

a) All the reaction were performed in ethyl acetate (100 ml) at25°C;diol 5 (25 mmol). b) 10 g of crude enzyme precipitated on 25 g of celite with 60% acetone in phosphate buffer pH 8. c) Same procedure as in ([b]) but using 50% acetone.

However, the optical purity of the recovered products was not satisfactory for our purposes. Since the commercially available PPL preparation is a mixture of different enzymes, it was decided to purify this crude preparation by fractional precipitation with acetone (ref. 8). The precipitated proteins were adsorbed on celite, dried and utilized in the transesterification reactions. The catalytic activity of this new enzymatic preparation was as good as that of crude PPL, but the stereoselectivity was much higher. As shown in table 2 the enantiomeric excess of the produced 6 changed from 53% to 95%. The optical purity of 6 was determined by ^{1}H-NMR of the corresponding MTPA (Mosher acid) ester. Optically pure 6 was transformed into (R)-4 and then in (R)-1 by reaction with toluene-4-sulfonyl chloride followed by reaction with the sodium salt of 1,2,4-triazole.

The key intermediate 7 was chosen as starting material in the synthesis of optically pure isomers of fungicide 2 because of the easy deprotection of the phenol moiety and because it could be used as a precursor of a wide class of fungicides carrying different substitution on the aromatic ring. As described for previous substrates, we compared the lipase catalyzed hydrolysis of the ester 8 to the lipase catalyzed transesterification of the alcohol 7.

Scheme 3



As reported in Table 3, PPL showed a very poor stereoselectivity in both the reaction conditions, whereas *Pseudomonas* lipase proved to be a much more selective catalyst in ethyl acetate (Scheme 3) than in water.

TABLE 3

SUBSTRATE	ENZYME	SOLVENT	CONV.%	ESTER 8		ALCOHOL 7	
				e.e.%	CONF.	e.e.%	CONF.
8	PL	H_2O [a]	45	22	R	30	S
8	PPL	H_2O	45	3	S	9	R
7	PL	AcOEt [b]	55	84	S	95	R
7	PL	AcOEt	45	98	S	79	R
7	PPL	AcOEt	60	13	R	15	S

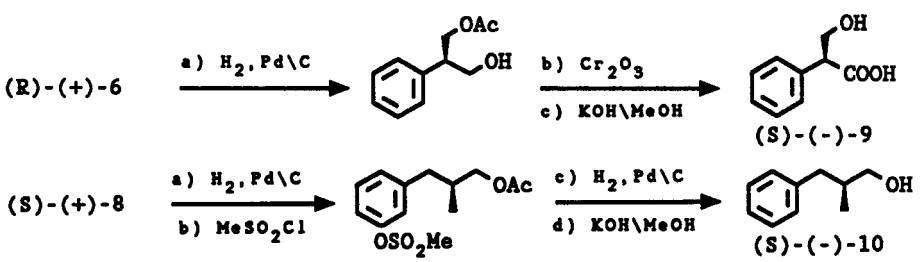
a) Hydrolytic reactions were performed in phosphate buffer 0.05 M, pH 7 (200 ml) at 30°C; substrate, 25 mmol; lipase, 500 mg. b) Transesterification reactions were performed in ethyl acetate (100 ml) at 25°C; substrate, 25 mmol; lipase, 1g.

This surprising result confirms the literature data reporting that enzymes stereoselectivity can be dramatically affected by the reaction medium (ref. 9). The transesterification reaction afforded the acetate 8 in the S form and the alcohol 7 in the R form. The optical purity of 7 was determined by HPLC analysis using a chiral column (Daicel Chiralcel OD). Enzymatically prepared 7 was then used for the synthesis of the four stereoisomers of fungicide 2. Substitution of the hydroxy group with iodine and deprotection of the phenol group was carried out simultaneously by reaction with trimethylchlorosilane and sodium iodide. Reactions with 2,6-dimethylmorpholine (mixture of cis- and trans-isomers) and with chloromethylcyclopropane afforded a mixture of cis- and trans-2. The two isomers were easily separated by silica gel chromatography. Starting from optically pure 7 all the stereoisomers (R)-(+)-cis-2, (R)-(+)-trans-2, (S)-(-)-cis-2 and (S)-(-)-trans-2, were prepared with enantiomeric excess ≥95%.

Determination of the absolute configuration

The absolute configurations of (+)- and (-)-1 were determined by chemical correlation with (R)-(+)-6, whose configuration was in its turn related to that of (S)-(-)-tropic acid (9)(Scheme 4).

Scheme 4



The absolute configuration of (+)- and (-)-2 were assigned by correlation with (S)-(+)-8, whose configuration was determined by chemical correlation with the known (S)-(-)-2-methyl-3-phenylpropan-1-ol (10) (Scheme 4).

BIOASSAY

The antifungal activity of racemic fungicide 1 and of the two enantiomers was assessed *in vitro* against a number of fungi (Table 4), and *in vivo* against *Erysiphe graminis* on wheat.

TABLE 4

PATHOGEN	EC_{50} VALUES(mg/l)			ACTIVITY RATIO
	(R,S)-1	(R)-1	(S)-1	(R/S)
Botrytis cinerea	2.1	1.2	23.5	19.6
Cercospora beticola	0.45	0.24	2.3	9.6
Guignardia bidwelli	0.035	0.02	0.12	6.0
Pyricularia oryzae	2.1	1.5	2.8	1.9
Sclerotinia minor	2.1	1.4	9.0	6.4
Sclerotium cepivorum	0.3	0.15	1.9	12.7
Cladosporium cucumerinum	0.17	0.11	0.85	7.7

Fungitoxicity tests were carried out in Petri dishes, with potato dextrose agar, by assessing the inhibition of mycelial radial growth of treated colonies with respect to untreated ones.

The *in vitro* tests showed that the (R)-(+)-isomer was always the more active one. *In vivo* activity against *E. graminis* was evaluated after one-day preventive treatment on artificially infected wheat plants. The results of these tests confirmed the *in vitro* data, showing an enantiomeric activity ratio (R isomer/S isomer) = 400.

The antifungal activity of racemic 2 and of its four stereoisomers was assessed *in vitro* against a serie of fungi (Table 5). As shown in table 5 *in vitro* tests indicate that cis\trans isomerism plays a more important role than R\S isomerism, in determining the fungitoxicity of the molecule. New and more detailed information will be available at the end of *in vivo* tests on fungicide 2, currently under investigation.

TABLE 5

PATHOGEN	EC_{50} VALUES(mg/l)					
	TRANS (R,S)-2	CIS (R,S)-2	TRANS (R)-2	TRANS (S)-2	CIS (R)-2	CIS (S)-2
Botrytis cinerea	2.0	1.1	6.0	2.0	1.1	1.1
Cercospora beticola	-	-	70.0	20.0	4.5	6.0
Cercosporella herpotrichoides	1.0	0.1	2.3	1.0	0.1	0.1
Septoria nodarum	-	-	0.6	0.6	0.6	0.6
Helminthosporium oryzae	-	-	3.4	1.4	0.3	0.3
Rhizoctonia solani	-	-	200.0	120.0	40.0	60.0

Fungitoxicity tests were carried out in Petri dishes, with potato dextrose agar, by assessing the inhibition of mycelial radial growth of treated colonies with respect to untreated ones.

In conclusion , this combined chemo-enzymatic approach provides a convenient method for the preparation of optically pure chiral fungicides, in quantities suitable for an accurate study of their biological activity. The preparation of new chiral synthons for the synthesis of optically active pesticides is currently under investigation.

Acknowledgement

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