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IDENTIFICATION OF $\Delta^{5,7}$-STEROL-Δ^{7}-REDUCTASE IN HIGHER PLANT MICROSOMES

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SUMMARY : A microsomal preparation from seedlings of *Zea mays* catalyzed the NADPH dependent reduction of the Δ^{7}-bond of $\Delta^{5,7}$-cholestadienol (1) giving the first *in vitro* evidence for the intermediacy of $\Delta^{5,7}$-sterols in plant sterol biosynthesis. Using a GC assay developed to detect the cholesterol (2) produced, the properties of the microsomal enzyme have been established with respect to cofactor requirements and kinetics. The potent *in vitro* inhibition of the plant $\Delta^{5,7}$-sterol-Δ^{7}-reductase by the ammonium-ion containing fungicides, tridemorph[2] (3), fenpropimorph (4) and AY 9944 (5) was demonstrated. The high affinities observed for these derivatives, especially for (4) ($I_{50} = 8\times10^{-8}$M, $I_{50}/K_m = 2\times10^{-4}$), are in full accordance with the previously proposed cationic mechanism involved in this reduction reaction.

During cholesterol biosynthesis in mammals, the Δ^{5}-bond is introduced via the sequence Δ^{7}-sterol $\rightarrow\Delta^{5,7}$-sterol $\rightarrow$ Δ^{5}-sterol. [1,2,3]. The existence of an enzyme (E.C.1.3.1.21) capable of reducing the Δ^{7}-bond of $\Delta^{5,7}$-cholestadienol has been demonstrated in rat liver homogenate [4] and has been partially purified [5]. $\Delta^{5,7}$-sterols have been rarely isolated in higher plants and very little is known of the pathway involved in the reduction of the Δ^{7}-bond in these organisms. $\Delta^{5,7}$-sterols have been reported in a few species such as bryophytes [6], *Ochromonas danica* [7] and lichens [8,9] strongly suggesting that they are intermediates in sterol biosynthesis in these organisms. In this investigation, we describe for the first time, a microsomal preparation isolated from a higher plant (*Zea mays*) which functions

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[2] Nomenclature used : tridemorph (3) : 4-(1,5,9-trimethyldecyl)-2,6-dimethyl morpholine ; fenpropimorph (4) : 4-[3-[4-tert-butylphenyl]-2-methyl]propyl-2,6-dimethyl morpholine ; AY9944.[(trans-1,4-bis(2-chlorobenzyl-aminoethyl)cyclohexane dihydrochloride)].

Abbreviations : GLC : gas liquid chromatography ; TLC : thin layer chromatography ; GLC-MS : coupled gas liquid chromatography-mass spectroscopy ; I_{50} : the inhibitor concentration which reduces the observed reaction rate by 50%. t_R : relative retention time to cholesterol.

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in the reduction of the Δ^7 bond of $\Delta^{5,7}$-cholestadienol. In addition, the reductase was also shown to be the target of a series of ammonium ion containing fungicides.

Materials and Methods

Chemicals and reagents

$\Delta^{5,7}$-cholestadienol was purchased from Fluka and crystallized from MeOH before use (m.p. 150°-151°C). $\Delta^{8,14}$-cholestadienol, $\Delta^{7,14}$-cholestadienol, Δ^7-cholestenol and Δ^8-cholestenol were synthetized as previously described [10]. Cholesterol was purchased from Fluka. The following chemicals and reagents were purchased from Sigma : NADPH (tetrasodium salt), βNADH (disodium salt), glucose oxidase (type V, from *Aspergillus niger*), glucose-6-phosphate dehydrogenase (type XV from bakers yeast), alcohol dehydrogenase (from yeast), glutathione (reduced form), Trisma base, glucose 6-phosphate. N-substituted morpholines were kindly provided by BASF Agrochemical Station (Limburgerhof, FRG). AY9944 was a generous gift from Dr Dvornik (Ayerst Research Laboratory,Montreal, Canada).

$\Delta^{5,7}$-Sterol Δ^7-reductase assay

Microsomes (pH 7.6) were prepared from maize seedlings as described previously [14,19]. In the standard procedure the microsomes (0.75 ml = 3 mg protein) were incubated in the presence of $\Delta^{5,7}$-cholestadien-3β-ol (200 μM) emulsified with Tween 80 (final concentration 1.5 g/l), 1 mM NADPH, 1 unit glucose-6-phosphate dehydrogenase, 10 mM glucose 6-phosphate and other additions as indicated in tables and figures. Incubations were continued at 30°C for 90 min with gentle stirring. The reaction was stopped by adding 1 ml 6% methanolic KOH, then extracted three times with hexane. After being dried with Na_2SO_4 the hexane phase was evaporated to dryness and the residue analysed by GLC using a silica-fused capillary column (WCOT, 25m x 0.25mm) coated with OV17, (230°C to 280°C, 2°/min) using hydrogen as carrier gas. $\Delta^{5,7}$-cholestadien-3β-ol (1) (t_R=1.088) and its reduction metabolites cholesterol (2) (t_R=1.000) were clearly separated on this column (Table 1, Fig.1). In addition they were well separated from the bulk of endogenous sterols : campesterol (t_R=1.129), stigmasterol (t_R=1.171) and sitosterol (t_R=1.241). The conversion ratio was calculated from the areas of the peaks of cholesterol (2) and untransformed compound (1) and corrected from endogenous components with corresponding t_R identical to the value obtained in the boiled assay (<0.06). The activity was obtained from the concentration of substrate and the conversion ratio.

In the case of inhibition assays, microsomes (0.75 ml) were incubated for 90 min at 30°C in the presence of (1) (200 μM) and increasing concentration of the inhibitor (6-10 different concentrations), from which dose-response curves were obtained allowing I_{50} value to be determined as described previously [10,12].

Protein was estimated according to Schacterle and Pollack [26] with BSA as a standard (Fraction V, Sigma Chemical Co).

Results and Discussion

$\Delta^{5,7}$-sterol-Δ^7reductase assay and product identification

During this initial study, $\Delta^{5,7}$-cholestadienol(1) was used as a potential substrate of Δ^7-reductase. By employing a substrate with a saturated side-chain, the putative reduction of side-chain unsaturation in our conditions was obviated. Moreover, using this sterol with a C8 side chain as substrate allowed a clearcut separation in GLC analysis of the residual substrate (1) and the expected product cholesterol (2) from the bulk of the endogenous sterols, namely campesterol (a), stigmasterol (b) and sitosterol (c), present in the membrane preparation.

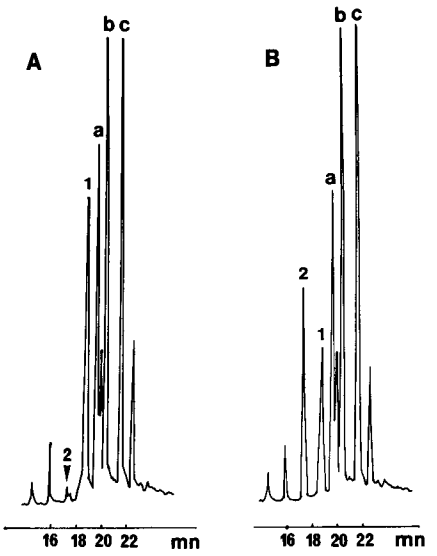

Fig. 1. Gas-liquid chromatographic assay for Δ^7-reductase.
Gas liquid chromatograms were obtained with an OV17-coated column and correspond to analysis of the neutral lipids fraction originating from incubation of $\Delta^{5,7}$-cholestadiene-3β-ol (1) with microsomes from maize embryos. A) Boiled microsomes ; B) 90 min incubation. Product is cholesterol (2). Endogenous sterols are essentially a) campesterol, b) stigmasterol and c) sitosterol.

Incubation of (1) with microsomes from corn (*Zea mays*) embryos in the presence of NADPH and a NADPH regenerating system produced a sterol metabolite (2) which was separated from the residual substrate (1) by capillary GLC (Fig. 1) and unequivocally identified as cholesterol (1) by coupled capillary GLC/MS analysis (i.e., by coincidental retention time and identical electron impact spectrum as that of an authentic standard) (Table 1).

Properties of microsome bound $\Delta^{5,7}$-sterol Δ^7-reductase (Δ^7-SR)

As shown in Table 2, $\Delta^{5,7}$-sterol-Δ^7-reductase exhibited an absolute requirement for NADPH. Substitution of NADPH by NADH resulted in unmeasurable rate of reduction, and addition of NADH to NADPH did not increase the reaction rate. A level of 1 mM NADPH with the inclusion of a regenerating system and of 200 µM of (1) sustained a linear reduction rate for at least 90 min (Fig. 2). Reaction rates were linear with respect to microsomal protein level up to 5 mg/ml (90 min assay) (Data not shown). With the aforementioned conditions, the velocity-substrates-concentration curves obeyed simple Michaelis-Menten kinetics leading to the apparent kinetic constants ; K_m and average V_{max} of the microsomal Δ^7-reductase obtained with $\Delta^{5,7}$-cholestadienol (1) were respectively 460 µM and 0.61 nmol.min^{-1}.mg protein^{-1} (Fig. 3A). K_m for NADPH was 10 µM and V_{max} was 0.2 nmol.min^{-1}.mg protein^{-1} under the highest soluble but non-saturating concentration of $\Delta^{5,7}$-cholestadienol, 200 µM (Fig. 3B).

The observed K_m value for (1) is significantly smaller than that obtained for the rat liver enzyme (2 mM) [4] but similar to that for the $\Delta^{8,14}$-sterol substrate of $\Delta^{8,14}$-sterol Δ^{14}-

Table 1. GC/MS[a] analysis of isolated substrate and isolated or anticipated metabolites of $\Delta^{5,7}$-sterol Δ^{7}-reductase

Compound	Rt relative to cholesterol in		percent relative abundance of molecular and prominent fragments ions m/e (%)					
	OV1	OV17						
Isolated compounds								
Isolated untransformed $\Delta^{5,7}$-cholestadienol (peak 1)	1.044[b]	1.088	384(84)	369(10) 271(11)	366(10) 253(19)	**351(100)** 211(13)	325(41) **158(16)**	**143(34**
Isolated enzymatically produced cholesterol (peak 2)	1.000	1.000	386(100)	371(39) 273(24)	368(59) 255(34)	353(48) 213(43)	301(40)	
Authentic standards								
$\Delta^{5,7}$-cholestadienol	1.044	1.088	384(84)	369(10) 271(11)	366(16) 253(19)	**351(100)** 211(12)	325(41) **158(16)**	**143(34**
$\Delta^{8,14}$-cholestadienol	1.012	1.046	384(100)	**369(88)** 271(18)	366(5) 256(7)	351(37)		
$\Delta^{7,14}$-cholestadienol	0.999	1.035	384(100)	369(33) **271(99)**	366(6) 253(18)	351(12)		
Cholesterol	1.000	1.000	386(100)	371(37) 273(24)	368(52) 255(25)	353(36) 213(36)	304(40)	
Δ^{7}-cholestenol	1 050	1 078	386(100)	371(27) 273(19)	368(5) **255(47)**	353(5)		
Δ^{8}-cholestenol	1 020	1.029	386(100)	371(37) 273(19)	368(14) 255(14)	353(11)		

a) 70 eV
b) S.D. = ± 0.0013 (n=5)
The values in **bold** type refer to characteristic fragmentations of the compounds [27].

reductase recently described in the same organism [10]. In addition the V_{max} value determined here for (1) and that obtained for the sterol substrate of $\Delta^{8,14}$-sterol Δ^{14} reductase in the same organism [10] are among the highest measured so far for post-squalene enzymes in higher plants [10-13] suggesting that these reduction steps are not rate determining in the sterol biosynthetic flux, in agreement with the rare isolation of $\Delta^{8,14}$ and $\Delta^{5,7}$-sterols in plants.

Table 2. Cofactor requirements of microsomal $\Delta^{5,7}$-sterol Δ^{7}-reductase

Incubation content	Relative reaction rate
Microsomes + 1 mM NADPH + RS_1[a]	100
Boiled microsomes + 1 mM NADPH + RS_1	0
Microsomes	0
Microsomes + 1 mM NADPH	83
Microsomes + 1 mM NADH + RS_2[b]	0
Microsomes + 1mM NADPH + 1 mM NADH + RS_1 + RS_2	104

a) RS_1 : NADPH regenerating system = Glu 6P (10 mM) + Glu 6P dehydrogenase (1 unit)
b) RS_2 : NADH regenerating system : EtOH + alcohol dehydrogenase (1 unit)

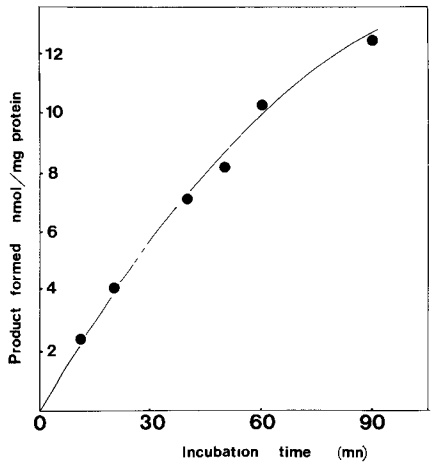

Fig. 2. Effect of incubation length on amount of product formed by $\Delta^{5,7}$-sterol Δ^{7}-reductase. The concentration of $\Delta^{5,7}$-cholestadienol (1) was 200 μM.

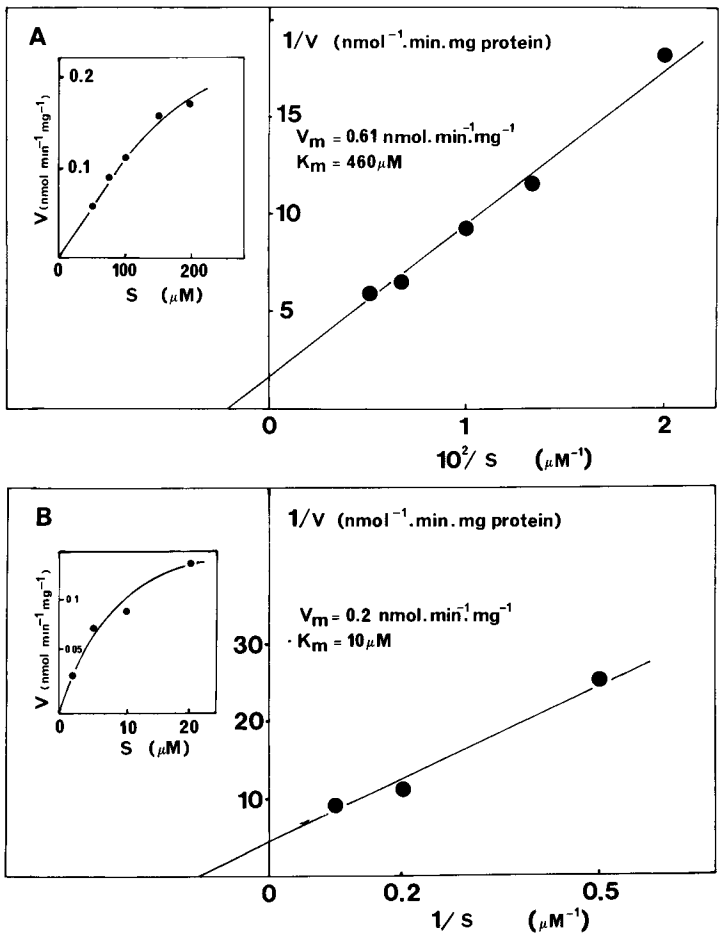

Fig. 3. Kinetics of $\Delta^{5,7}$-sterol Δ^{7}-reductase. A : Lineweaver-Burk double-reciprocal plot for $\Delta^{5,7}$-cholestadienol (1) ; the concentration of NADPH was 1 mM (with addition of a regenerating system). B : Lineweaver-Burk double reciprocal plot for NADPH (with addition of a regenerating system) ; the concentration of (1) was 200 μM.

Inhibition of plant $\Delta^{5,7}$-sterol Δ^{7}-reductase by ammonium-ion-containing fungicides

During the last decade it has been shown that an important group of fungicides acting as inhibitors of sterol biosynthesis are N-alkyl morpholine derivatives such as tridemorph (3) or fenpropimorph (4) [14,15]. These compounds were also demonstrated to be inhibitors of sterol biosynthesis in higher plants interfering both *in vitro* and *in vivo* with cycloeucalenol-obtusifoliol isomerase (COI) [16], $\Delta^{8}\rightarrow\Delta^{7}$-sterol isomerase ($\Delta^{8}\rightarrow\Delta^{7}$SI) [17] and $\Delta^{8,14}$-sterol Δ^{14}-reductase (Δ^{14} SR) [10]. The protonated form of these derivatives was shown to be responsible for the inhibition [17], suggesting these compounds to model the C8, C9 and C14 high energy cationic intermediates (HEI) respectively involved in the pathway of these three reactions. Results of mechanistical studies performed with mammalian systems have shown that Δ^{7}-reductase also involves a HEI, with positive charge localized at C7 which is then neutralized by the delivery of a hydride ion from NADPH [18] (Fig. 4). Thus it was of interest to know whether N-alkyl morpholines could also bind to the plant Δ^{7}-reductase. To find out, these last derivatives were directly assayed *in vitro* with the plant enzyme.

Results from Table 3 clearly indicate that fenpropimorph (4) is a potent inhibitor of plant microsomal $\Delta^{5,7}$-sterol-Δ^{7}-reductase (I_{50} = 8×10^{-8}M ; I_{50}/K_m = 2×10^{-4}). Furthermore the enzymatic activity is less but still sensitive to tridemorph (3) and AY 9944 (5), a potent inhibitor of the animal $\Delta^{5,7}$-sterol-Δ^{7}-reductase activity [19,2]. A similar rational as that proposed previously for the potent inhibition of COI, $\Delta^{8}\rightarrow\Delta^{7}$ SI and Δ^{14} SR by (3) and (4) [10,16,17] could be proposed for the observed potent inhibition of Δ^{7}-SR by these derivatives, in accordance with the suggested mechanism, that is to say : the protonated form of these compounds would interact with the active site domain which stabilizes the putative C7 carbenium ion intermediate involved in the Δ^{7}-double bond reduction process. The relative

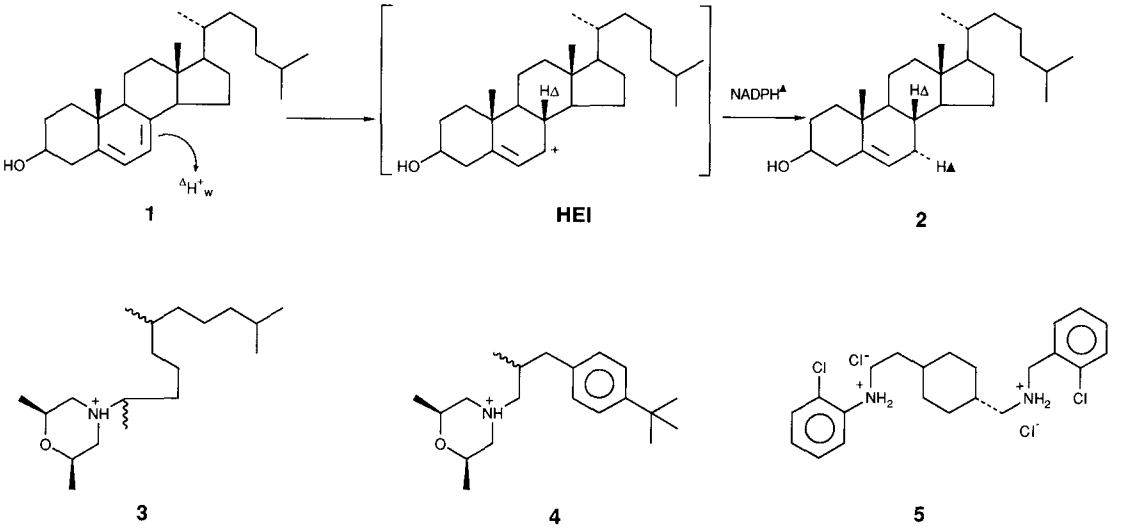

Fig. 4. Reaction pathway for $\Delta^{5,7}$-sterol Δ^{7}-reductase and chemical structures of the inhibitors considered in the present work.

Table 3. Comparative inhibition of $\Delta^{5,7}$-sterol Δ^7-reductase (Δ^7 S.R.), $\Delta^{8,14}$-sterol Δ^{14}-reductase (Δ^{14}SR), $\Delta^8 \rightarrow \Delta^7$-sterol isomerase ($\Delta^8 \rightarrow \Delta^7$ SI) and cycloeucalenol-obtusifoliol isomerase (COI) by ammonium ion-containing fungicides

	I_{50} (μM) with compound		
Enzyme	3	4	5
Δ^7 SR[a]	0.5	0.08	1.2
Δ^{14}SR[b]	25	0.8	40
Δ^8-Δ^7 SI[b]	0.4	0.4	0.5
COI[c]	0.4	0.4	N.I.

[a] This work
[b] Data from [10]
[c] Data from [20]
N.I. not inhibitor (<5% inhibition at 200 μM).

weakness of inhibition of Δ^7-reductase by (3) in comparison to (4) suggests the presence of a specific apolar binding site for the N-alkyl substituent. Similar results were previously obtained for inhibition of plant Δ^{14}-SR whereas Δ^8-Δ^7-SI and COI showed identical affinities for (3) or (4) indicating that this last binding site could be linked to the NADPH dependent ene-reductase activity. Furthermore, results from Table 3 clearly show that the same morpholinium derivative is able to mimic four different HEI's with sp^2 carbon atoms in the adjacent C-7, C-8, C-9 and C-14 positions. The reasons for these unique multi-target inhibitory properties of (3) and (4) are probably twofold : Firstly they could be due to the structural flexibility of the hydrophobic substituent linked to the morpholinium pharmacophore. This flexibility would lead to the ability to be accomodated by the active site domains of the four aforementioned enzymes normally occupied by the C, D rings and side-chain of the substrate. Second, as previously discussed [10], this could reflect a delocalized charge in such ammonium ions between the nitrogen and the adjacent carbon and hydrogen atoms, inside the active sites, a medium of probably low dielectric constant [20].

The potent *in vitro* inhibition of Δ^{14} SR, $\Delta^8 \rightarrow \Delta^7$-SI and COI by (3-5) has been previously fully correlated with their *in vivo* effects on sterol profile following the treatment of plants with these compounds [16,21]. However, treatment of plants with (3) and (4) did not led to accumulation of $\Delta^{5,7}$-sterols indicating that Δ^7-SR is not the main target of (3) and (4) *in vivo* [16,21]. This is probably due to the aforementioned concomitant strong inhibition of COI, $\Delta^8 \rightarrow \Delta^7$-SI and Δ^{14}-SR by these derivatives thus blocking the biosynthetic flow upstream and preventing accumulation of the substrate of Δ^7-SR situated the farthest downstream. In accordance with this hypothesis a notable accumulation of $\Delta^{5,7}$-sterols (together with a strong accumulation of Δ^8-sterols) was found following the treatment of maize seedlings with (5) [22] which is shown in the present work to interfere with a more restricted number of targets, i.e.

Δ^8-Δ^7-SI and Δ^7-SR. However, in some other cases, only a strong accumulation of Δ^8-sterols could be observed *in vivo* following treatment of plants cells with (5) [23,24]

Finally, the new enzymatic assay developed in the present investigation constitutes a new tool for searching novel potent inhibitors specifically targeted towards plant Δ^7-SR. The *in vivo* treatment of plants with such compounds could lead to the replacement of typical plant Δ^5-sterols by typical fungal $\Delta^{5,7}$-sterols and thus would give another opportunity to explore the function of sterols in plant membranes [25].

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