Properties and structural requirements for substrate specificity of cytochrome *P*-450-dependent obtusifolios 14 α -demethylase from maize (*Zea mays*) seedlings

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The biochemical properties of cytochrome *P*-450-dependent obtusifolios 14 α -demethylase (*P*-450_{OBT·14DM}) from maize (*Zea mays*) seedlings were defined. In particular, the enzyme was shown by differential centrifugation to be localized in the endoplasmic reticulum. *P*-450_{OBT·14DM} had an apparent K_m of $160 \pm 5 \mu\text{M}$ and an apparent V_{max} of $65 \pm 5 \text{ pmol/min per mg of protein}$ for its best substrate, obtusifolios. The substrate specificity of *P*-450_{OBT·14DM} was thoroughly investigated by comparing the demethylation of obtusifolios with that of a series of 15 natural or novel synthetic analogues of obtusifolios. The results obtained clearly indicate that three distinct domains of the sterol substrate are governing obtusifolios demethylation by *P*-450_{OBT·14DM}. They revealed that (i) *P*-450_{OBT·14DM} has probably a specific apolar binding site for the side chain, (ii) the Δ^8 -double bond is an absolute requirement for substrate demethylation and (iii) the 3-hydroxy group plays a critical role in the enzyme–substrate interaction. Interestingly the binding site, beyond the C-3 position, contains a cleft which cannot accommodate a 4 β -methyl substituent present in lanosterol or eburicol, the precursors of 14-desmethylsterols respectively in mammals and yeast. This result indicates that *P*-450_{OBT·14DM} is a novel constitutive cytochrome *P*-450 with a high degree of substrate and product specificity.

INTRODUCTION

Biotransformation of endogenous and exogenous compounds in higher plants represents a developing field of high biological relevance. However, our knowledge of the enzymology of plant sterol biosynthesis still reveals large gaps. Moreover, the number of cytochrome *P*-450 (*P*-450)-dependent oxygenases which have been identified in higher plants is still limited, and the function and specificity of *P*-450s are much less well known than in animals and fungi, probably because of the low content and supposed lability of *P*-450 in cell-free plant systems. Plant *P*-450s are involved mainly in secondary metabolism [1,2]. They are also suspected of participating in a number of reactions associated with xenobiotic metabolism [3] in a similar way to the numerous mammalian liver *P*-450s involved in detoxification processes [4]. Finally, the occurrence of analogous *P*-450-dependent reactions in different organisms may lead to attractive comparative studies.

We have recently described a microsomal preparation isolated from a higher plant (maize, *Zea mays*) that is functional in the removal of the 14 α -methyl group of obtusifolios (1, Fig. 1) which is metabolized to the corresponding 4 α -methyl-5 α -ergosta-8,14,24(28)-trien-3 β -ol (17) [5] (Fig. 1 and Scheme 1). To our knowledge this enzyme constitutes with digitoxin 12 β -hydroxylase from the foxglove, *Digitalis lanata* [6], the only two *P*-450-dependent hydroxylases of steroid-like molecules which have

been described so far in higher plants. In the present work we have further defined some biochemical properties of this enzyme. In addition, with the aim of providing insight into the mechanism and specificity of this new plant *P*-450, we have begun studies to probe the interaction of the enzyme with its substrate. Thus we have prepared, and in a number of cases synthesized, a wide range of potential substrates possessing a 14 α -methyl group and different chemical structure in the side chain, steroid nucleus or the C-3-position domain. The results enabled us to delineate remarkably strict structural requirements governing substrate demethylation.

RESULTS AND DISCUSSION

Properties of the microsome-bound 14 α -demethylase

Subcellular localization. The subcellular distribution of obtusifolios 14 α -demethylase (*P*-450_{OBT·14DM}) was studied by differential centrifugation of the crude homogenate isolated from *Zea mays* embryos. More than 98 % of the demethylase activity and of the *P*-450 content were associated with the 100 000 *g* pellet corresponding to the microsomal fraction, whereas no activity could be detected in the 100 000 *g* supernatant. After elimination of mitochondria in a 10 000 *g* pellet, resolution of the 100 000 *g* pellet into several membrane fractions by linear sucrose-density-

Abbreviations and trivial names used: t_R , retention time; TMS, tetramethylsilane; TBDMS, t-butyldimethylsilyl; DMF, dimethylformamide; MCPBA, metachloroperbenzoic acid; THF, tetrahydrofuran; *P*-450, cytochrome *P*-450; *P*-450_{OBT·14DM}, cytochrome *P*-450-dependent obtusifolios 14-demethylase; HMPA, hexamethylphosphoramide; ER, endoplasmic reticulum; PM, plasma membrane; AdoMet-CMT, S-adenosyl-L-methionine: cycloartenol C-24-methyltransferase; UDPG-SGT, UDP-glucose:sterol glucosyltransferase; the systematic names for the sterols are as follows: obtusifolios (1, see Fig. 1), 4 α ,14 α -dimethyl-5 α -ergosta-8,24(28)-dien-3 β -ol; cycloeculenol (5), 4 α ,14 α -dimethyl-9 β ,19-cyclo-5 α -ergosta-24(28)-en-3 β -ol; 31-norlanosterol (4), 4 α ,14 α -dimethyl-5 α -cholesta-8,24-dien-3 β -ol; 24-methylenelophenol (19), 4 α -methyl-5 α -ergosta-7,24(28)-dien-3 β -ol; eburicol (or 24-methylenelanosterol) (9), 4,4,14 α -trimethyl-5 α -ergosta-8,24(28)-dien-3 β -ol; lanosterol (13), 4,4,14-trimethyl-5 α -cholesta-8,24-dien-3 β -ol; 14-desmethyl-lanosterol, 4,4-dimethyl-5 α -cholesta-8,24-dien-3 β -ol; fecosterol, 5 α -ergosta-8,24(28)-dien-3 β -ol; 24(25)-dehydrolophenol, 4 α -methyl-5 α -cholesta-7,24(25)-dien-3 β -ol; 14-desmethyl-31-norlanosterol, 4 α -methyl-5 α -cholesta-8,24(25)-dien-3 β -ol; cycloartenol, 4,4,14-trimethyl-9 β ,19-cyclo-5 α -cholesta-24(25)-en-3 β -ol.

Fig. 1. Chemical structures of the substrates and products considered in the present work

The numbers given are referred to in the text.

Scheme 1. Proposed pathway for the conversion of obtusifoliol (1) to 24-methylenelophenol (19) by *Zea mays* microsomes

gradient centrifugation according to procedures previously described for plant membranes [7,8] allowed a light membrane fraction [density (ρ) 1.10 g/ml] and a heavy membrane fraction (ρ 1.17 g/ml) to be isolated (Table 1). NADPH:cytochrome *P*-450 (cytochrome *c*) reductase activity, a classical marker of ER, and AdoMet-CMT, an enzyme involved in free sterol biosynthesis and previously localized in the ER [8], were shown to be mainly associated with the light fraction (Table 1), indicating the presence in this fraction of membranes derived from the ER. The occurrence of an important UDPG-SGT activity in the heavy fraction suggests that this fraction is enriched in PM vesicles [8]. From results in Table 1 it appears that totality of *P*-450 content and *P*-450_{OBT-14DM} activity are localized in the light membrane fraction enriched in membranes derived from ER. This location is consistent with that found for most of cytochrome *P*-450 and associated monooxygenase activities identified so far in plant

tissues [3,7,9–11]. Moreover, it is identical with that previously found for NADPH:cytochrome *c* reductase and a series of other enzymes involved in post-squalene sterol biosynthesis in *Zea mays* [8].

Determination of kinetic parameters. All the following experiments were performed by using the standard enzymic assay and the microsomal pellet, which was sedimentated at 100 000 *g* for 1 h.

Under the standard assay conditions (50 μ M in obtusifoliol [5]), the rate of demethylation of (1) was constant for incubation periods up to 4 h of duration, and up to 6 h for a concentration of (1) of 100 μ M, after which the reaction rate decreased slowly (results not shown). This period is much longer than that observed in the case of the yeast (about 5 min at 30 °C) or the rat liver (60 min at 37 °C) enzymes, indicating a remarkable stability of the plant demethylase. Furthermore, under these conditions the

Fig. 2. Kinetics of *P*-450_{OBT-14DM} for selected substrates
●, Obtusifoliol (1); ○, 24(25)-dihydro-31-norlanosterol (3).

Fig. 3. pH profile of microsomal *P*-450_{OBT-14DM}
Symbols: ▲, 0.1 M-sodium phosphate buffer; ●, 0.1 M-Tris/HCl buffer.

amount of (1) metabolized was proportional to the amount of microsomal proteins up to 5 mg·ml⁻¹ (results not shown).
With the aforementioned conditions, the velocity–substrate-concentration curves obeyed simple Michaelis–Menten kinetics

Table 1. Subcellular localization of the 14 α -demethylase activity

Component (units)	Fraction ... ρ (g/ml) ...	ER 1.10	PM 1.17
Protein (mg·ml ⁻¹)		2.6	1.4
NADPH:cytochrome <i>c</i> (cytochrome <i>P</i> -450) reductase (μ mol·min ⁻¹ ·mg ⁻¹)		43	2.0
AdoMet-CMT (nmol·h ⁻¹ ·mg ⁻¹)		2.5	0.15
UDPG-SGT (nmol·h ⁻¹ ·mg ⁻¹)		0.35	3.3
Cytochrome <i>P</i> -450 (pmol·mg ⁻¹)		228	21
<i>P</i> -450 _{OBT-14DM} (nmol·h ⁻¹ ·mg ⁻¹)		0.73	nd*

* nd, not detectable (estimated limit of detection for the enzyme activity 0.04 nmol·h⁻¹·mg⁻¹).

Fig. 4. Model of structural requirements for substrate demethylation
The rectangles and parallelograms define essential domains of obtusifoliol governing interaction with *P*-450_{OBT-14DM}.

Table 2. Structural requirements for demethylation by *P*-450_{OBT-14DM}

All substrates or analogues were assayed at a concentration of 50 μ M. Obtusifoliol was incubated in parallel in each experiment and its rate of demethylation taken as 100 %. Results are means for two to four experiments. ‘N.S.’ means that the sterol is not a substrate, i.e. the rate inferior or equal to the estimated limit of detection for enzyme activity (0.04 nmol·h⁻¹·mg⁻¹).

Sterol substrate	Rate	
	(nmol·h ⁻¹ ·mg ⁻¹)	(%)
Obtusifoliol (1)	1.20 ± 0.06	100
24 ζ (28)-Dihydro-obtusifoliol (2)	0.90 ± 0.06	75
24,(25)-Dihydro-31-norlanosterol (3)	0.80 ± 0.08	67
31-norlanosterol (4)	N.S.	0
Cycloeucalenol (5)	N.S.	0
4 α ,14 α -Dimethyl-5 α -ergost-9(11)-en-3 β -ol (6)	N.S.	0
4 α ,14 α -Dimethyl-5 α -ergost-7-en-3 β -ol (7)	N.S.	0
8(9),24(25)-Tetrahydro-31-norlanosterol (8)	N.S.	0
24-Methylenelanosterol (9)	N.S.	0
24 ζ (28)-Dihydro-4 β -methyl-30-norobtusifoliol (10)	N.S.	0
14 α -Methyl-24 ζ (28)-dihydrofecosterol (11)	0.60 ± 0.06	50
24,(25)-Dihydrolanosterol (12)	N.S.	0
Lanosterol (13)	N.S.	0
Obtusifoliyl-3 β -methoxy (14)	N.S.	0
Obtusifoliyl-3 β -acetoxy (15)	N.S.	0
Obtusifoliyl-3 β -amino (16)	N.S.	0

Table 3. G.c.-m.s. analysis of substrates and metabolites of *P-450*_{OBT-14DM}The values in **bold** type refer to characteristic fragmentations of the compounds [52].

Substrate	<i>m/z</i> (relative intensity)							
	Substrate		Metabolites					
Substrate	Δ^8 -14 α -Methylsterol		$\Delta^{8,14}$ -Dienesterol	Δ^8 -14-Desmethylsterol		Δ^7 -14-Desmethylsterol		
Obtusifolol (1) (analysis performed with 3 β -acetoxy derivatives)	468 (50)	(1) 453 (100)	393 (41)	(17) 452 (100) 377 (29) 311 (19) Isolated under interrupted conditions* Not isolated under standard incubation conditions Not isolated under standard incubation conditions Not isolated under standard incubation conditions	(18) 439 (20) 327 (20) 454 (100) 379 (17) 227 (22)	454 (25) 327 (100) 241 (11)		
	287 (25)	241 (13)	409 (33) 326 (28)				396 (16) 241 (27)	439 (17) 269 (21) 227 (16)
24 ζ (28)-Dihydro- obtusifolol (2)	428 (22) 395 (14)	(2) 413 (100)	410 (4)		(22) 399 (26) 269 (13)	396 (18) 227 (24)	(23) 399 (26) 269 (31)	
24 ζ (25)-Dihydro-31- norlanosterol (3)	414 (24) 365 (4) 245 (9)	(3) 399 (100) 341 (2) 227 (5)	381 (16) 259 (6)		(20) 385 (18) 287 (9) 243 (10)	382 (9) 269 (7) 227 (12)	385 (22) 287 (13) 243 (10)	
					(24) 385 (25) 273 (61) 213 (22)	382 (11) 229 (25)	385 (24) 273 (25) 213 (17)	
14 α -Methyl-24 ζ (28)- dihydrofecoesterol (11)	414 (19) 381 (12) 245 (5)	(11) 399 (100) 365 (2) 227 (4)	397 (8) 341 (2)					

* In the presence of 10 μ M-*N*-benzyl-4 α ,10-dimethyl-8-aza-*trans*-decal-3 β -ol *N*-oxide, an inhibitor of *Δ*^{8,14}-sterol-*Δ*¹⁴-reductase [5,29].

in accordance with the participation of a single *P*-450 enzyme (active site) in the oxidative demethylation of obtusifolioside. However, the adoption of formulae applicable to single-phased enzyme systems led only to apparent values of K_m and V_{max} , since these values are probably depending on the membrane concentration in the microsomal system used. Thus all experiments described herein were performed at constant protein and detergent concentrations (see the Experimental section). The apparent K_m and V_{max} of the microsomal *P*-450_{OBT-14DM} were determined with obtusifolioside (1) and 24(25)-dihydro-3 β -lanosterol (3), another good substrate of the enzyme. K_m and V_{max} obtained respectively from a double-reciprocal plot were $160 \pm 5 \mu M$ and $65 \pm 5 \text{ pmol} \cdot \text{min}^{-1} \cdot \text{mg of protein}^{-1}$ for (1) and $116 \pm 5 \mu M$ and $40 \pm 5 \text{ pmol} \cdot \text{min}^{-1} \cdot \text{mg of protein}^{-1}$ for (3) (Fig. 2).

These observed K_m values are somewhat higher than those obtained for the rat liver enzyme ($50 \mu M$) [12,13] or the *Candida albicans* (yeast) enzyme ($53 \mu M$) [14], tested with 24(25)-dihydro-3 β -lanosterol in microsomal preparations.

Optimization of pH for catalysis. The effect of pH on enzymic activity was determined over the pH range 6.5–9.5 (Fig. 3). The optimal pH range for the demethylation of obtusifolioside was found to be between 7.5 and 8.5.

Structural requirements for substrate specificity of *P*-450_{OBT-14DM}

Assuming that demethylation of substrate and analogues results from interactions with a common catalytic site and that the partition coefficients of (1) and analogues between buffer and membranes are identical, we have examined the interaction of obtusifolioside (1) with *P*-450_{OBT-14DM}. Thus we compared the maize microsomal demethylation of (1) with that of 15 other 14 α -methylsteroid analogues differing in a single structural feature (Fig. 1). The original synthesis of a number of these analogues is described in the Experimental section. The ability of these analogues to be demethylated was assayed in the same *Zea mays* microsomal preparation using an identical concentration of each compound. This concentration ($50 \mu M$) was chosen as it leads to a high conversion ratio of substrate into products (see the Experimental section) and thus favours the detection, under our assay conditions, of possibly poorly metabolized analogues. However, in the case of metabolized substrates (1, 2, 3 and 11), the relative reaction rates obtained with higher substrate concentrations (close to the K_m value) compared closely with those obtained with a $50 \mu M$ concentration (results not shown). A micellar dispersion of Tween 80 in water was used as a carrier for the insoluble substrates or inhibitors on the microsomal membranes as described previously [5]. Under standard conditions where obtusifolioside was converted in 40–50% ratio to Δ^8 and Δ^7 metabolites (18) and (19) (30 °C, 4 h) we examined the conversion ratio of these various analogues after adaptation, if necessary, of the conventional work-up (see the Experimental section). Comparison of the reaction rates allowed the features of principal importance for substrate demethylation to be determined. From the results obtained it has been possible to determine three distant domains essential in the substrate transformation by *P*-450_{OBT-14DM}. Fig. 4 summarizes the portions of obtusifolioside controlling its interaction with *P*-450_{OBT-14DM}.

Influence of the structure of the apolar side chain. A series of obtusifolioside analogues differing from one another in the structure of the non-polar side chain have been assayed as potential substrates for *P*-450_{OBT-14DM}. Results in Table 2 show that, surprisingly, the nature of the substitution at C-24 is critical for substrate demethylation. Indeed, introduction of a $\Delta^{24(25)}$ double bond, leading to the more rigid isopropylidene moiety in comparison with the flexible iso-octane or 24-methyl iso-octane side

chain without a $\Delta^{24(25)}$ double bond, reproducibly precluded 14 α -demethylation (within the limit of detection for the enzyme activity) and led to the complete recovery of the untransformed substrate. In contrast, the presence of a saturated side chain with or without a C-24 substituent only slightly lowered its metabolism by *P*-450_{OBT-14DM}. These results show the importance of both the structure and the flexibility of the non-polar side chain for demethylation and suggest that the *P*-450_{OBT-14DM} has a specific apolar binding site for the side chain of obtusifolioside.

Influence of the steroid nucleus structure. In a similar series of investigations, the rate of reaction of a variety of substrate analogues with different steroid nucleus structures, related to the presence and localization of the steroid unsaturation, was examined. Data from Table 2 show that no analogues possessing a 9 β ,19-cyclopropane ring (5), a $\Delta^{9(11)}$ (6), a Δ^7 (7) double bond or a completely saturated nucleus (8), were metabolized under our experimental conditions.

Thus the presence of a $\Delta^{8(9)}$ unsaturation in the substrate is compulsory for catalysis. This could reflect the greater reactivity of Δ^8 substrates, leading to conjugated diene products, a process which could provide part of the driving force for the final lytic reaction. However, conformational and steric requirements affecting the orientation of the 14 α -methyl group are probably also important, since the Δ^7 ,14 α -methylsteroid (7) is not demethylated. This last result is in contrast with the observed demethylation of 14 α -methylsteroids with a Δ^7 double bond by the demethylase from rat liver [15,16], and suggests a stronger substrate specificity of the plant demethylase. Incidentally, double-bond regio-specificity has been recently shown for another enzyme in the plant sterol biosynthetic pathway, namely Δ^8 ,14-sterol 14-reductase [17]. The placement of the double bond in the Δ^8 position was also compulsory for catalysis in that case.

Influence at substituents of C-4 and role of the 3 β -hydroxy group. A series of C-4 position analogues was examined in order to assess the recognition by *P*-450_{OBT-14DM} of the substrate methyl group to this position. A variety of differently 4-substituted obtusifolioside or 24(28)-dihydro-obtusifolioside analogues was used, since we have shown that reduction of the methylene substituent has minor effects on the metabolic rate. Results from Table 2 clearly indicate that the presence of a 4 β -methyl group, in addition to, or in place of, the 4 α -methyl group present in (1) [compounds (9) and (10)] totally precludes demethylation, leading to complete recovery of the untransformed substrate within the limits of detection for the enzyme activity (see the Experimental section). In contrast, absence of a methyl group at position C-4 only slightly hindered enzymic demethylation, since 14 α -methyl-24(28)-dihydrofecosterol (11) was metabolized to the expected Δ^8 - and Δ^7 -14-desmethyl products (see the Experimental section and Table 3). Taken together, these results suggest a means of substrate C-4 methyl-group recognition which could be a sterically constrained cleft above the C-4 position.

The effect of changes at the 3-position of obtusifolioside on substrate demethylation was examined by using a series of differently 3-substituted obtusifolioside analogues. From the result shown in Table 2 it is apparent that the 3 β -hydroxy group is critical for the substrate to be transformed, since none of the 3-substituted analogues listed is metabolized by *P*-450_{OBT-14DM} under our experimental conditions. The absence of transformation of the 3 β -methoxy (14) and the 3 β -acetoxy (15) derivatives suggests engagement of the 3 β -hydroxy group in hydrogen-bonding with some subsite of the active site, which would be sensitive to steric hindrance by groups attached to the oxygen atom. In addition, the absence of transformation of the isosteric, but more basic, 3 β -amino analogue (16), would exclude hydrogen-bonding of the hydrogen atom from the 3-hydroxy substituent with a basic residue of the active site. However, the

inertness of this compound could also be related to its possible protonation, leading to other interactions, such as electrostatic repulsion, with the active site. Thus the presence of the 3 β -hydroxy group might be necessary for proper orientation of (1) in the substrate-binding pocket.

The metabolism of substrate analogues different from the physiological substrate have led to different conclusions regarding the mechanism of C-14 α -demethylation in animals [18] and, in particular, regarding the involvement of $\Delta^8(14)$ -ene [19,20] or $\Delta^8,14$ -diene intermediates [12,21,22]. In the present case, results show that no trace of $\Delta^8(14)$ isomer could be detected during metabolism of obtusifoliosol (1) as well as its analogues (2), (3) and (11).

In accordance with our previous work [5], the demethylation of (1), (2), (3) and (11) by the maize microsomal-bound 14 α -demethylase was totally dependent upon NADPH, whereas NADH proved to be an extremely poor reductant (results not shown). This result is in contrast with that obtained with rat liver demethylase, which could be supported by addition of NADH, suggesting the involvement of a cytochrome b_5 -dependent electron transfer in addition to the NADPH-supported pathway [12]. A similar exclusive NADPH-dependence has been described for cinnamate hydroxylase, another plant *P*-450-dependent mono-oxygenase [7,9].

Taken together, the results of the structure-activity relationship described herein show that, for the majority of analogues assayed, all-or-none reactivities were obtained, suggesting absolute structural requirements. Such essential requirements may be necessary for the orientation of the 14 α -methyl group directly above the haem iron for efficient and regio-specific C-32 hydrogen abstraction followed by stereospecific oxygen rebound [23]. In addition, the apparent complete recovery of substrate analogues which are not demethylated after incubation indicates that the initial oxidative step of the demethylation process is hampered by the structural modifications. Moreover this complete recovery clearly shows no alteration of the regioselectivity of the demethylase towards these analogues. This result would be in contrast with the observed alteration of regioselectivity obtained with other *P*-450-dependent hydroxylations when confronted with substrate analogues [24].

The observed strict specificity is consistent with the constitutive nature of *P*-450_{OBT-14DM}, as opposed to detoxification *P*-450s and associated mono-oxygenase activities, and provides a further argument for a plurality of specific *P*-450 and associated mono-oxygenase activities in higher plants. Animal and yeast demethylases were shown to efficiently demethylate lanosterol and its 24(25)-dihydro derivative, whereas both these 4,4-dimethylsterols were not transformed by the plant enzyme, indicating clearly that the plant demethylase is a distinct enzyme. Our results raise the question of whether this surprising difference of substrate specificity indicates the presence of large differences in amino acid sequence and structure of these different *P*-450s. However, strong variation of substrate specificity may result from limited amino acid replacements insofar as they are located in the immediate vicinity of the bound substrate. Indeed recent work has shown the feasibility of altering *P*-450 substrate specificity, regioselectivity or properties via mutation of a single or a few amino acid residues [24–27].

The observed substrate selectivity of the plant demethylase sustains biosynthetic data, which suggest that, in contrast with animals and fungi, in higher plants elimination of the 14 α -methyl group would be processed only after elimination of the 4 α -methyl group and cleavage of the 9 β ,19-cyclopropane ring [28]. Indeed, inhibition of cycloeucalenol:obtusifoliosol isomerase led *in vivo* to accumulation of 9 β ,19-cyclopropyl-14 α -methylsterols [29]. In addition, our results are consistent with the general

finding that naturally occurring $\Delta^9(11)$ -4-desmethylsterols generally possess a 14 α -methyl group [30–32]. In this connection it is important to mention that *Zea mays* has been shown *in vivo* to transform not only labelled cycloeucalenol, but also lanosterol, into β -sitosterol [36]. Similar results were obtained with other species of higher plants [37,38]. These '*in vivo*' results contrast with the present findings showing the absence of C-14 demethylation of substrates possessing a 4 β -methyl substituent and the absence of '*in vitro*' C-4 demethylation of lanosterol by the same cell-free system (S. Pascal, M. Taton & A. Rahier, unpublished work). Moreover, the present work clearly shows the absence of C-14 demethylation of 31-norlanosterol. They would also contrast with the exclusive accumulation of Δ^8 -14 α -methylsterols possessing no 4 β -methyl group after the treatment of maize seedlings *in vivo* with potent inhibitors of *P*-450_{OBT-14DM} [39]. This apparent discrepancy between approaches using whole organisms on the one hand, and '*in vitro*' enzyme systems on the other, could reflect the absence of detection, during assays *in vitro*, of trace levels of products resulting from an extremely low rate of metabolism when compared with that of the physiological substrate (< 1/100), owing to the inherent limit of sensitivity of these assays. In contrast, long-time feeding of abnormal precursors *in vivo* may reveal kinetically slow and hidden metabolic pathways which are not followed in the normal biosynthetic situation. In addition, during '*in vivo*' feeding experiments of large hydrophobic sterol structures using whole plants, differentiation between low and high rates of incorporation of distinct precursors might be hampered by many other, non-biosynthetic biochemical mechanisms (i.e. uptake, transport etc.) slower than the biosynthetic steps to be studied. From this point of view, feeding experiments would be inadequate at revealing precisely the structural requirements. Finally, the strict substrate specificity of *P*-450_{OBT-14DM} constitutes another example of an obligatory or all-or-none sequence of intermediates involved in plant sterol biosynthesis, in agreement with previous findings [33,34].

A key problem in the design of fungicides, herbicides, plant growth regulators and drugs acting as inhibitors of *P*-450-dependent reactions is selectivity. *P*-450_{OBT-14DM} is the target of important agrochemicals such as fungicides, plant growth regulators and herbicides [35], which co-ordinate with the *P*-450 iron atom, and are also supposed to interact with the substrate-binding domain. The results of the present study could provide part of the ground-work needed to be able to design specific competitive inhibitors of plant *P*-450_{OBT-14DM}. They could also be useful in the design of agrochemical fungicides, interacting with the substrate binding domain of the fungal *P*-450 dependent-lanosterol 14 α -demethylase, which would not bind to the obtusifoliosol binding site of plant *P*-450_{OBT-14DM}. In this last case, plants treated with such compounds could be prevented from the phytotoxic effects owing to the blockade of plant sterol biosynthesis leading to replacement of Δ^5 -sterols by 14 α -methyl- Δ^8 -sterols.

EXPERIMENTAL

Materials

The following chemicals and reagents were purchased from Sigma: NADPH (tetrasodium salt), β -NADH (disodium salt), glucose oxidase (type V, from *Aspergillus niger*), glucose-6-phosphate dehydrogenase (type XV from baker's yeast), GSSG, Trizma base and glucose 6-phosphate.

UDP-[U-¹⁴C]glucose and *S*-adenosyl-L-[methyl-³H]methionine were purchased from Amersham International.

Chemical details

Melting points are uncorrected. M.s. and g.c.-m.s. were determined at 70 eV with an LKB 9000F spectrometer. The g.c. separation was carried out on a capillary column [25 m long $\times$ 0.25 mm internal diameter (i.d.)] coated with OV17. G.c. analysis was carried out with a g.c. instrument equipped with a flame-ionization detector and a fused-silica capillary column [WCOT; 25 m long $\times$ 0.25 mm (i.d.)] coated with OV1 or OV17 (H_2 flow 2 ml/min). The temperature program used included a fast rise from 60 to 240 °C (30 °C/min), then a slow rise from 240 to 280 °C (2 °C/min). Relative retention times (t_R) are given with respect to cholesterol ($t_R = 1$). P.m.r. was monitored in a [^2H]chloroform solution with a Bruker 200 MHz or 400 MHz spectrometer. Chemical shifts (δ) (p.p.m.) were determined relative to tetramethylsilane.

Substrate analogues

All compounds which have been previously synthesized were shown to have > 98% g.c. purity and analytical data in accordance with their structure and the reported literature data.

24 ζ (28)-Dihydro-obtusifolioside (2), m.p. 124–125 °C, (recrystallized from methanol) and 4 β ,14 α (24 ζ)-28-trimethyl-5 α -cholest-8-en-3 β -ol (10) were synthesized as described previously [40].

24(25)-Dihydro-31-norlanosterol (3) was synthesized as described previously [33], m.p. 115–117 °C (methanol).

31-Norlanosterol (4) was kindly supplied by Professor R. Goutarel (Institut de Chimie des Substances Naturelles, Gif-sur-Yvette, France), m.p. 109–111 °C (methanol) (literature value 110 °C [41]).

24-Methylenlanosterol (9) was generously given by Dr. R. Brandt (Hoffmann-La Roche, Basel, Switzerland) (m.p. = 147–150 °C, acetate derivative recrystallized from methanol).

14-Desmethyl-31-norlanosterol and 14-desmethyl-lanosterol were generously given by Dr. J. L. Gaylor (E. I. du Pont de Nemours and Co., Wilmington, DE, U.S.A.).

Cycloeucalenol (5) was extracted as described previously [42] from tallow wood (*Eucalyptus myrcocorys*) kindly provided by Professor R. A. Massy Westropp (University of Adelaide, Adelaide, Australia), m.p. 139–140 °C (methanol).

14 α -Methyl-24 ζ (28)-dihydrofosterol (11). This compound was extracted and purified from maize seedlings treated with an inhibitor of obtusifolioside 14 α -methyl demethylase as described previously [39]. It showed g.c. purity > 98%; (OV17, $t_R = 1.179$) g.c.-m.s.: m/z (relative intensity) M^+ (the molecular ion) = 414(19), 399(100), 397(8), 381(12), 365(2), 341(2), 245(5), 227(4). ^1H -n.m.r. [400 MHz (^2H)chloroform]: δ 0.715 (3 H, s, H-18), 0.786 (3 H, d, J 7 Hz, H-28), 0.789 (0.4 $\times$ 6 H, d, J 7 Hz, H-26–27, 24 R diastereoisomer), 0.812 (0.6 $\times$ 6 H, d, J 7 Hz, H-26–27, 24 S diastereoisomer), 0.859 (0.6 $\times$ 3 H, d, J 7 Hz, H-21, 24 S diastereoisomer), 0.864 (0.4 $\times$ 3 H, d, J 7 Hz, H-21, 24 R diastereoisomer), 0.893 (3 H, s, H-32), 0.952 (3 H, s, H-19), 3.625 (1 H, td, J 11 Hz, J 5 Hz, H3 α).

24(25)-Dihydrolanosterol (12) and lanosterol (13) were purchased as a '50/50' mixture from Sigma. Both these compounds were separated, on t.l.c. plates impregnated with $AgNO_3$, as their acetate derivatives. Saponification and recrystallization from methanol yielded pure crystals (12), m.p. 144–146 °C and (13) m.p. 138–139 °C.

4 α ,14 α -Dimethyl-5 α -ergost-9(11)-en-3 β -ol (6) was synthesized as described previously [43] by HCl isomerization of (24 ζ), (28)-dihydrocycloeucalenol, m.p. 117.5–118.5 °C (methanol).

4 α ,14 α -Dimethyl-5 α -ergost-7-en-3 β -ol (7). A mixture (20 mg) of 4 α ,14 α -dimethyl-5 α -ergost-8-en-3 β -ol (2) (20%) and (7) (80%) was synthesized as described previously [43] by HCl

isomerization of 24 ζ (28)-dihydrocycloeucalenol (m.p. 117.5–118.5 °C, methanol). (7) was purified from the mixture by an h.p.l.c. instrument equipped with an SiO_2 μ Porasil (3.9 mm $\times$ 300 mm) column and eluted with a mixture of hexane/*t*-butyl methyl ether (99.6/0.4, v/v) leading to pure (7), which was recrystallized from methanol: m.p. 175–176 °C. (7) Showed a g.c. purity > 95%; (OV17). M.s.: m/z (relative intensity) $M^+ = 428(23)$, 413(100), 395(38). ^1H n.m.r. [400 MHz, (^2H)chloroform]: δ 0.658 (3 H, s, H-18), 0.782 (3 H, d, J 6.9 Hz, H-28), 0.784 (3 H, d, J 6.8 Hz, H-27, 24 S -diastereoisomer), 0.805 (3 H, d, J 6.8 Hz, H-27, 24 R -diastereoisomer), 0.843 (3 H, s, H-19), 0.852 (0.5 $\times$ 3 H, d, J 7.1 Hz, H-21, 24 R -diastereoisomer), 0.858 (3 H, d, J 6.5 Hz, H-26 R), 0.879 (3 H, d, J 6.5 Hz, H-26 S), 0.888 (0.5 $\times$ 3 H, d, J 6.3 Hz, H-21, 24 S -diastereoisomer), 0.981 (3 H, s, H-14), 0.983 (3 H, d, J 6.3 Hz, H-30), 3.119 (1 H, dt, J 10 Hz, J 3.4 Hz, H3 α), 5.178 (1 H, dm, J 5.8 Hz, H-7).

Synthesis of 4 α ,14 α -dimethyl-5 α -cholestanol (8). Attempts to reduce the Δ^8 double bond of 24(25)-dihydro-31-norlanosterol (3) using sodium hexamethylphosphoramide (HMPA) and *t*-butyl alcohol [44] were unsuccessful; thus the next procedure was followed: 24(25)-dihydro-31-norlanosterol (3) (200 mg) was silylated according to the procedure of Hosoda *et al.* [45], yielding 4 α ,14 α -dimethyl-5 α -cholest-8-en-3 β -*t*-butyldimethylsilyl (TBDMS) ether (26) (210 mg). This last derivative (200 mg), in dry chloroform (30 ml), was treated with a vigorous stream of dry HCl for 20 min at 20 °C and left in chloroform saturated with HCl for 3 h. The mixture was diluted with water, basified with aq. NH_3 and extracted with hexane. After washing with water, drying over anhydrous Na_2SO_4 , the extract was evaporated to dryness and the products analysed by g.c.-m.s. as their TBDMS ether derivatives, yielding a mixture of 4 α ,14 α -dimethyl-5 α -cholest-8-en-3 β -TBDMS ether and 4 α ,14 α -dimethyl-5 α -cholest-7-en-3 β -TBDMS ether (27) (80%). (M.s. and n.m.r. data were consistent with the proposed structures.)

A solution of the above dry mixture (80 mg) dissolved in dry tetrahydrofuran (THF) (4 ml) was then added to a blue solution of sodium metal ($\approx$ 100 mg) in a mixture of dry HMPA (20 ml) and *t*-butyl alcohol (1.5 ml) under argon as described by Whitesides & Ehmman [44]. The mixture was stirred at room temperature for 14 h under argon; during this period the blue colour of the HMPA solution never completely disappeared. The mixture was then poured into an ice/water mixture and extracted with hexane. The organic phase was then evaporated to dryness. In order to separate the Δ^8 isomer that had not reacted, the residue (37 mg) was dissolved in diethyl ether (5 ml) and allowed to react with *m*-chloroperbenzoic acid (150 mg) for 14 h at 25 °C, leading to epoxidation of the unreduced Δ^8 isomer [43]. After usual work-up the reaction mixture was purified by t.l.c. using hexane/ethyl acetate (7:3, v/v) as migrating solvent. Thus the separation of 4 α ,14 α -dimethyl-5 α -cholest-8,9-epoxy-3 β -ol (28) ($R_F = 0.28$) (the 3 β -TBDMS ether was cleaved during the reductive procedure) from pure 4 α ,14 α -dimethyl-5 α -cholestan-3 β -ol ($R_F = 0.45$) (8) was achieved [10 mg; m.p. 145–147 °C (methanol)]. (8) gave a single peak on g.c.; m.s.: m/z (relative intensity): $M^+ = 416(19)$, 401(6), 383(3), 276(42), 261(100), 243(15), 233(27), 220(11), 215(6), 192(92). ^1H n.m.r. [400 MHz, (^2H)chloroform]: δ 0.778 (6 H, d, J 5.5 Hz, H-26 or H-27), 0.850 + 0.856 + 0.866 + 0.872 (12 H, H-18, H-19, H-32, H-21), 0.947 (3 H, d, J 6.3 Hz, H-30), 3.072 (1 H, td, J 5 Hz, J 10 Hz, H3 α).

The reduction by electrons of the starting 9 α , Δ^7 isomer (27) was assumed to yield the 9 α ,8 β B/C-ring *trans* junction, since the C-8 intermediate anion (or perhaps its precursor, the radical) will adopt the conformation of lowest energy before protonation [46, and references cited therein].

3 β -Amino-obtusifolioside (16) (amorphous compound), obtusifolioside 3 β -acetate (15) (m.p. = 106–107 °C, methanol) and obtusi-

foliyl 3 β -methyl ether (14) (m.p. = 89.5–91.5 °C, methanol) were synthesized as described previously [33].

Obtusifoliol synthesis

24-Oxo-4 α ,14 α -dimethyl-5 α -cholest-8-en-3 β -TBDMS ether. 31-Norlanosterol (4) (1.05 g) was silylated as described by Hosoda *et al.* [45], yielding 4 α ,14 α -dimethyl-5 α -cholest-8,24-dien-3 β -TBDMS ether (29) (1.1 g), m.p. 120–122 °C (dichloromethane/methanol). A portion (1.05 g) was dissolved in dry THF (20 ml) under argon and treated with a 1 M solution of borane in THF (5.5 ml) for 4 h at room temperature. The excess reagent was then destroyed with water (500 μ l); 3 M-NaOH (1 ml) and H₂O₂ (30 % in water) (6 ml) were then added and allowed to react for 1 h at room temperature. Water was added and the mixture extracted with hexane, which was dried over anhydrous MgSO₄. The resulting residue (1.1 g) was added to a solution of dipyridine-chromium(VI) oxide (3.5 g) in dichloromethane (20 ml) and allowed to react for 15 min at 25 °C under argon. Usual work-up yielded a residue (920 mg) which was recrystallized from methanol/dichloromethane, yielding 24-oxo-4 α ,14 α -dimethyl-5 α -cholest-8-en-3 β -TBDMS ether (30) (600 mg) as white needles (m.p. 125–127 °C). The compound showed a g.c. purity of > 95 %. M.s. and n.m.r. data for (29) and (30) were consistent with the structures proposed.

Obtusifoliol (1). To a solution of methyltriphenylphosphonium bromide in anhydrous benzene (20 ml) was added a solution of *n*-butyl-lithium in hexane (1.6 M, 800 μ l). The mixture was stirred for 15 min at 25 °C under argon. Then a solution of (30) (130 mg) in dry benzene (5 ml) was added dropwise under argon. The mixture was allowed to react for 30 min at 20 °C and stopped by the addition of diethyl ether saturated with water (5 ml). Usual work-up afforded a residue which was purified by t.l.c. using hexane/ethyl acetate (19:1, v/v) as eluent. The fraction with R_F 0.8 yielded pure obtusifoliol-3 β -TBDMS ether (31) (110 mg), which was recrystallized from methanol/dichloromethane (m.p. 159–161 °C, needles). The compound showed a single peak on g.c. analysis (OV1 or OV17): m/z (relative intensity): M^+ = 540(22), 525(20), 483(55), 407(7), 393(26). ¹H n.m.r. [200 MHz, (²H)chloroform] δ 0.711 (3 H, s, H-18), 0.886 (3 H, s, H-32), 0.901 + 0.938 (15H, two singlets, *t*-butyl + H-30 + H-21), 0.970 (3 H, s, H-19), 1.035 (6 H, d, *J* 6.5 Hz, H-26 + H-27), 3.078 (1 H, td, *J* 10 Hz, *J* 4.5 Hz, H3 α), 4.672 [1 H, s, W_1 (half-height width of the peak) = 4 Hz, H-28E], 4.721 (1 H, s, H-28Z). (31) (100 mg) was deprotected using tetrabutylammonium fluoride in THF, yielding pure obtusifoliol (1) (78 mg), which was recrystallized from methanol (m.p. 139–141 °C; literature value 144 °C [47]). The compound showed a g.c. purity of > 98 % (OV17, t_R = 1.200). G.c.-m.s.: m/z (relative intensity): M^+ = 426(14), 411(100), 408(4), 393(18), 383(7), 327(5), 313(8), 245(25), 215(8).

Plant material

Maize (variety LG11) seedlings were allowed to germinate in moist vermiculite for 60 h at 25 °C. The seedlings were harvested and the embryos separated from the caryopses.

Standard assay of P-450_{OBT-14DM}

Microsomes from *Zea mays* were prepared as previously described [5]. They were incubated aerobically as described previously [5] in the presence of obtusifoliol (1) (50–100 μ M) or various substrate analogues (50 μ M).

Incubation of substrate analogues

In the case of incubations of substrate analogues the assay procedure was adapted using the following modifications: incubation of analogues (2–16) was performed in the same way as for (1), but KOH was not used to stop the reaction in the case of (15). The other difference was the choice of the t.l.c. migrating solvent used for separation of the mixture of substrate and expected products from other constituents of the microsomal lipid extract. The presence or not of a 14 α -methyl group and the localization or number of unsaturations was previously shown not to affect the R_F of sterols on silica-gel plates with the solvent used herein [25,29,33]. Thus the migrating solvent used and the R_F values on t.l.c. for the different substrate analogues and expected products were as follows: compounds (1–8), (17–23): dichloromethane; two migrations; R_F = 0.24; compounds (9), (12), (13): dichloromethane; two migrations; R_F = 0.28; compound (10): dichloromethane; two migrations; R_F = 0.18; compounds (11), (24), (25), dichloromethane; two migrations; R_F = 0.14; compound (14): dichloromethane; one migration, R_F = 0.40; compound (15), dichloromethane; one migration; R_F = 0.49 and compound (16) (acetamide derivative), chloroform/methanol (19:1, v/v); one migration; R_F = 0.49. In the case of (11) the 4-desmethylsterol fraction eluted from the silica gel was further purified by t.l.c. on AgNO₃ impregnated alumina using chloroform/hexane/acetone (12:6:1, by vol.) as migrating solvent. $\Delta^8 + \Delta^7$ sterols (R_F = 0.38) were readily separated from endogenous Δ^5 sterols (R_F = 0.14) in this system.

The fraction co-migrating with the authentic standard of substrate and expected to contain reaction products was scraped off and analysed by g.c. using a glass capillary column [WCOT; 25 m long $\times$ 0.25 mm (i.d.)] coated with OV17 (240–280 °C at 2°/min) using H₂ as carrier gas. Under these conditions, substrate and metabolites obtained [in cases where the substrate was transformed, i.e. (1), (2), (3) and (11)] were readily separated [48,49]. Moreover, in the case of untransformed substrates, such as (4) and (13), the corresponding expected Δ^8 -14-desmethyl and Δ^7 -14-desmethyl analogues were shown to be readily separated under these chromatographic conditions. The t_R values relative to cholesterol on the OV17 column for the corresponding 14 α -methyl Δ^8 -, Δ^8 -14-desmethyl- and Δ^7 -14-desmethyl-sterols were as follows: obtusifoliol series: (1) 1.202, (18) 1.219, (19) 1.292; 24 α (28)-dihydro-obtusifoliol series: (2) 1.178, (22) 1.197, (23) 1.266; 24(25)-dihydro-31-norlanosterol series: (3) 1.061, (20) 1.071, (21) 1.126; 31-norlanosterol series: (4) 1.152, authentic standard of 14-desmethyl-31-norlanosterol, 1.168, authentic standard of 24(25)-dehydrolophenol, 1.234; 14 α -methyl-24 α (28)-dihydrofesterol series: (11) 1.179, (24) 1.197, (25) 1.243, lanosterol series: (13) 1.281, authentic standard of 14-desmethyl-lanosterol, 1.315. The reproducibility of these t_R values under identical analytical conditions was very good: S.D. = $\pm$ 0.002 (n = 4). The conversion ratio was calculated from the areas of the peaks of products and remaining substrate, corrected from endogenous components of same t_R with the values obtained in the boiled assay and normalized to the conversion ratio obtained for (1) in the same experiment. In the case of (4)–(10) and (12)–(15) (50 μ M), the assay (4 h incubation at 30 °C) and the boiled assay gave identical g.c. profiles in accordance with a conversion ratio of less than 2 %, whereas (1) (50 μ M) was converted into (18) and (19) with an average 50 % conversion ratio under these conditions. In the case of untransformed substrates, even a longer incubation (14 h) did not allow any product to be detected. In addition, in that case, a thorough g.c.-m.s. analysis of the assay using ion-monitoring recorded at M^+ , M^+ -CH₃ and other characteristic fragmentations [52] of the substrate and the expected products of the demethylation reaction

was carried out. This allowed us, when compared with the 'boiled' assay, to confirm the complete recovery of the untransformed substrate analogue within the limits of the experimental error. In the case of (16) the KOH/methanol-treated microsomes were extracted with hexane and the extract was directly acetylated with acetic anhydride/pyridine (1:1, v/v) for 14 h at 20 °C. After freeze-drying of the reactants, the residue was purified by t.l.c. (dichloromethane/methanol, 19:1, v/v) and the fraction with $R_F = 0.49$, co-migrating with a standard of 3 β -acetamido-obtusifolioside, was scraped off and analysed by g.c. as described above, which allowed a good separation of such derivatives [33]. In that case also, no significant conversion of (16) could be observed, the g.c. profile corresponding to that of the 'incubated' assay being qualitatively and quantitatively identical with that of the boiled assay.

Isolation of membranes

Membrane fractions were isolated by differential and isopycnic sucrose-density-gradient centrifugation as described previously [7,8]. Two purified membrane fractions were used in the present work: a light fraction (density 1.10 g/ml) rich in endoplasmic reticulum (ER) and a heavy fraction (1.17 g/ml) mainly consisting of plasma-membrane (PM) vesicles. Membranes were re-suspended in 0.1 M-potassium phosphate buffer, pH 7.5, containing 20% (v/v) glycerol, 1 mM-GSSG, 1 mM-EDTA and 4 mM-MgCl₂. Specific subcellular components (ER, PM) were identified by assays for the following markers: ER, NADPH: cytochrome *P*-450 (cytochrome *c*) reductase, *S*-adenosyl-L-methionine: cycloartenol C-24-methyltransferase (AdoMet-CMT) [8,50]; PM, UDP-glucose:sterol glucosyltransferase (UDPG-SGT).

Enzyme assays

NADPH:cytochrome *P*-450 (cytochrome *c*) reductase was measured spectrophotometrically [50]. AdoMet-CMT was assayed as described previously [51]. The cycloartenol concentration used was 50 μ M and the *S*-adenosyl-L-methionine concentration 100 μ M. UDPG-SGT was assayed as described previously [53]. Membrane protein was determined as described by Schacterle and Pollack [54], with BSA as a standard (fraction V; Sigma Chemical Co.). The cytochrome *P*-450 content in microsomes or membrane fractions was determined spectrophotometrically from its dithionite (Na₂S₂O₄)-reduced CO difference spectrum using absorption-coefficient difference between 450 and 490 nm of 91 mm⁻¹·cm⁻¹ [55].

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