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INHIBITION OF HIGHER PLANT 2,3-OXIDOSQUALENE CYCLASES BY NITROGEN-CONTAINING OXIDOSQUALENE ANALOGUES

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Key Word Index—*Pisum sativum*; *Zea mays*; Leguminosae; Graminae; pea; maize; biosynthesis; sterols; oxidosqualene cyclase; inhibition.

Abstract—10-Aza-10,11-dihydro-2,3-oxidosqualene (10 N-OS), 19-aza-18,19,22,23-tetrahydro-2,3-oxidosqualene (19 N-OS) and 23-aza-22,23-dihydro-2,3-oxidosqualene (23 N-OS), designed as analogues of carbocationic intermediates involved in the reaction pathway of 2,3-oxidosqualene-cycloartenol cyclase (OSCC) and/or 2,3-oxidosqualene-β-amyrin cyclase (OSβAC), and which could possibly be cyclase activated, were shown to be novel inhibitors of both OSCC and OSβAC in maize and pea microsomes. 10 N-OS had low activity on both plant cyclases with an I_{50} varying from 60 to 200 μM. 19 N-OS was active on both OSCC (I_{50} = 40 μM) and OSβAC (I_{50} = 15 μM), but there was no evidence of enzyme activation. 23 N-OS was also an efficient inhibitor of OSβAC (I_{50} = 1 μM) and OSCC (I_{50} = 3 μM), suggesting that it presumably acts as a mimic of the initial C-2 carbocation intermediate involved in the cyclization catalysed by both cycloartenol- and β-amyrin-cyclases.

INTRODUCTION

2,3-Oxidosqualene cyclases (EC 6.5.99.8) are key enzymes in the biosynthesis of sterols. They catalyse the cyclization of (3*S*)-2,3-oxidosqualene into lanosterol in non-photosynthetic organisms [1, 2] and into cycloartenol and a variety of pentacyclic triterpenoids such as β-amyrin in photosynthetic organisms [3, 4]. This enzyme has generally not been found in prokaryotic organisms (bacteria and cyanobacteria) which do not contain sterols [5]. The chemistry and enzymology of these complex ring-forming reactions has been reviewed recently [6].

In higher plants, results of recent biosynthetic studies strongly suggest that 2,3-oxidosqualene-cycloartenol cyclase (OSCC) and 2,3-oxidosqualene-β-amyrin cyclase (OSβAC) are regulatory steps in the isoprenoid pathway orienting the biosynthetic flux towards either phytosterols or pentacyclic triterpenes, which are the precursors of a large variety of physiologically active compounds [7, 8]. Besides the challenge of its intricate mechanism, 2,3-oxidosqualene cyclase (OSC) is also an interesting target for manipulating sterol contents in animal and plant cells. A large variety of inhibitors that are potentially useful as hypocholesterolaemic, antifungal or phototoxic drugs have been developed [9–11].

We have previously shown that carbenium ion high energy intermediates (HEIs) involved in the mechanism of sterol processing enzymes could be successfully mimicked by tailor-made azasteroids and simpler aza-derivatives [12–15]. It was first shown in our laboratory and then in others that acyclic, monocyclic, bicyclic and tricyclic compounds possessing a nitrogen atom at C-2, *pro*-C-8, *pro*-C-10 and *pro*-C-13, respectively, corresponding to the carbenium ion of HEIs or transition states formed during cyclization of 2,3-oxidosqualene, were potent OSCC inhibitors [16–21]. Recently, acyclic nitrogen-containing oxidosqualene (OS) analogues, i.e. 10-aza-10,11-dihydro-2,3-oxidosqualene (**1**) [22, 23], 19-aza-18,19,22,23-tetrahydro-2,3-oxidosqualene (**3**) [24] and 23-aza-22,23-dihydro-2,3-oxidosqualene (**6**) [25], were designed and synthesized as potential cyclase-activated HEI analogues to mimic, respectively, the *pro*-C-8 and *pro*-C-20 HEI involved in the pathway of both OSCC and OSβAC, and the C-23 lupenyl cation (Scheme 1) involved only in that of OSβAC. They were expected to bind the OS-cyclases in the ground state and to progress rapidly, with possible partial cyclization by the OS-cyclase, to the conformation complementary to the HEI. Indeed, **1**, **3** and **6** were shown to have various activities on different mammalian and fungal OS-cyclases [22–25].

We report in the present paper that **1**, **3** and **6**

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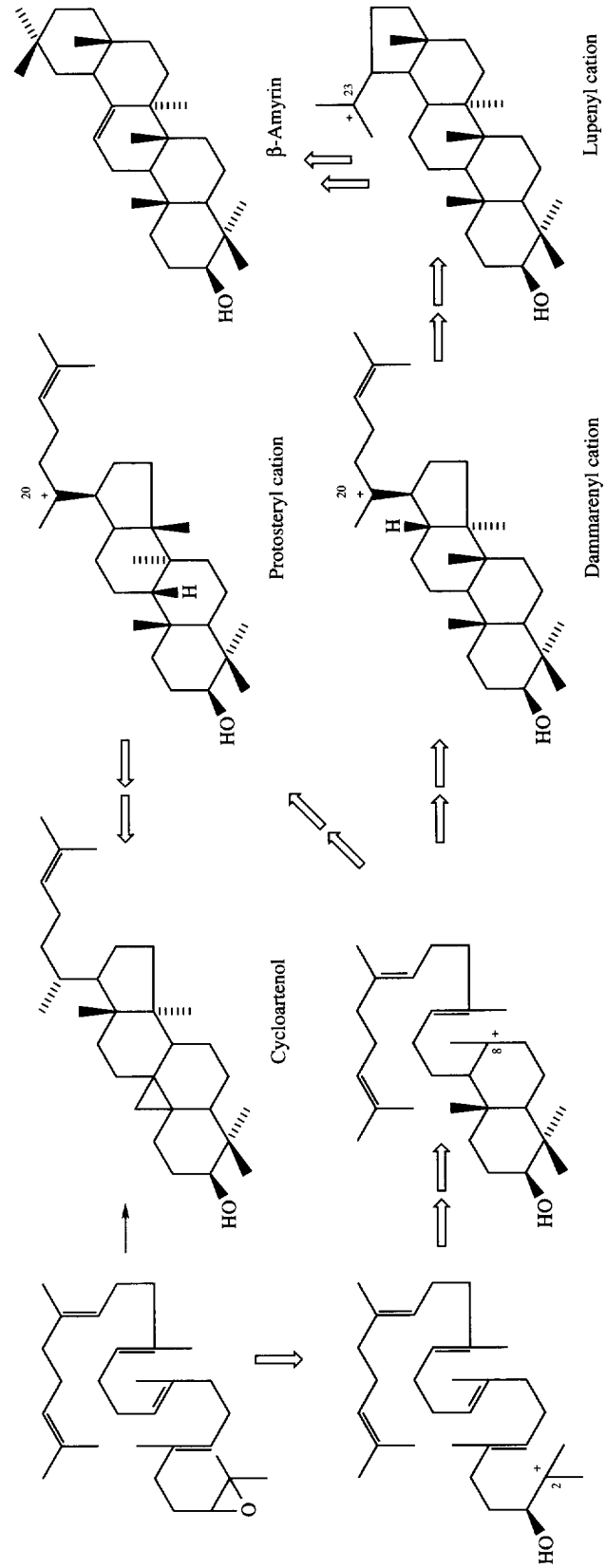

Scheme 1. Summarized postulated mechanism of 2,3-oxidosqualene-cycloartenol- and β-amyrin-cyclases.

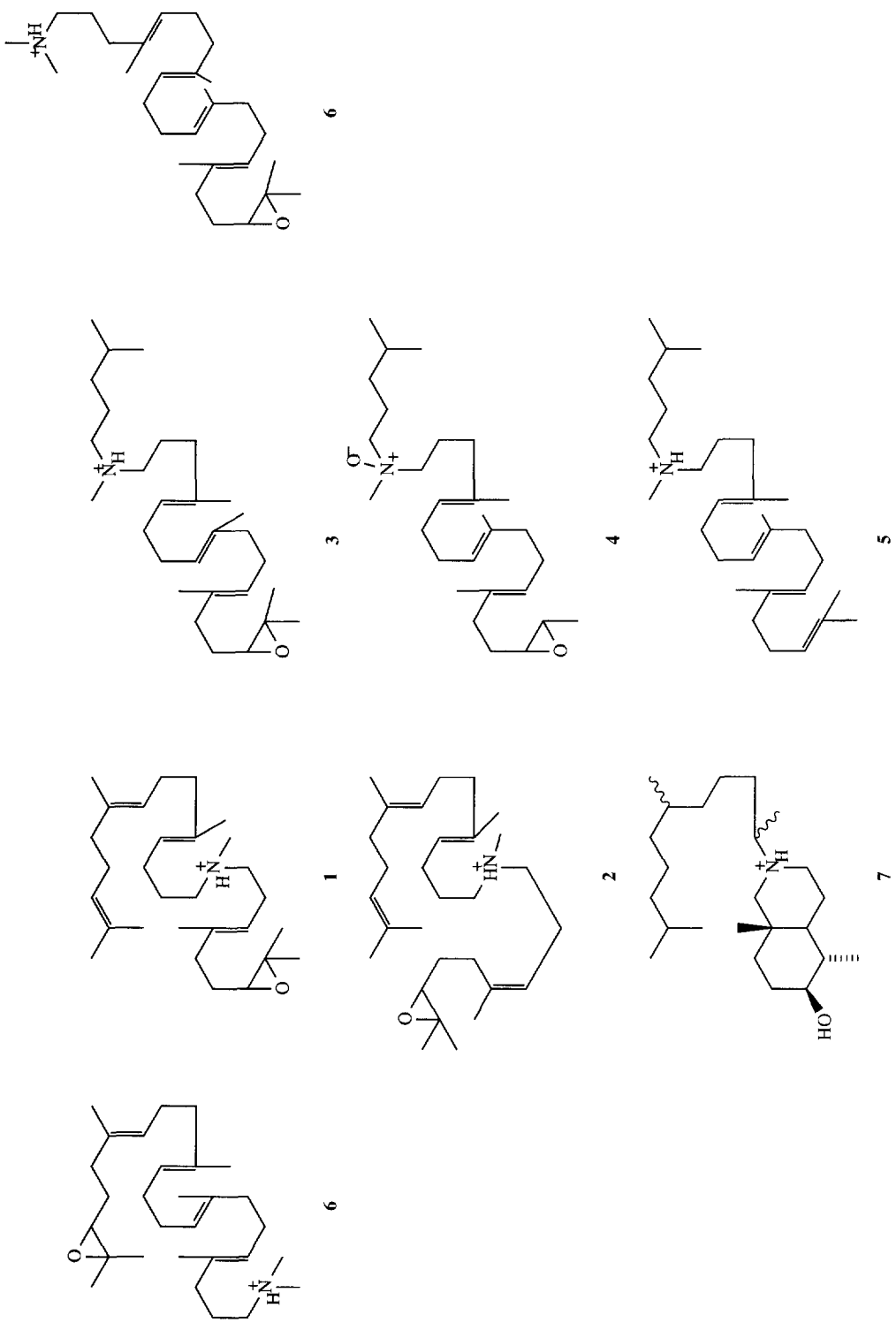

Fig. 1. Chemical structures of nitrogen-containing oxidosqualene analogues used in the present study.

Table 1. Inhibition of 2,3-oxidosqualene cyclases by various nitrogen containing oxidosqualene analogues

Enzyme	I_{50} (μM) with compounds 1	2	3	4	5	6	K_m for oxidosqualene (μM)
Maize embryo OSCC	60	70	40	50	40	3	125
Pea cotyledon OSβAC	100	200	15	100	40	1	125
Pea embryos: ratio of (α + β)-amyrins versus cycloartenol formed in presence of inhibitor (concentration used, μM)	nd* —	nd —	2.3 (30)	2.6 (100)	2.0 (100)	1.65 (30)	2.0 (without inhibitor)

*Not determined.

constitute novel inhibitors of plant OS-cyclases, i.e. OSCC from maize embryos and OSβAC from pea cotyledons.

RESULTS AND DISCUSSION

The inhibitory properties of **1**–**6** were determined *in vitro* without preincubation on the activity of microsomal OSCC from maize (*Zea mays*) seedlings and OSβAC from germinating pea (*Pisum sativum*) cotyledons. In addition these compounds were assayed on microsomes from pea seedlings which contain both OSCC and OSβAC. The data (Table 1) show that the diverse nitrogen-containing OS analogues significantly inhibited the different cyclase activities. However, they differ greatly in their affinity for the cyclases with I_{50} values varying from 1 to 200 μM (Fig. 2). Nevertheless, most of these values were below the K_m of 2,3-oxidosqualene for both cyclases (125 μM). 6*E*-10-Aza-10,11-dihydro-2,3-oxidosqualene (**1**) was moderately active on OSCC and relatively poorly active on OSβAC. In addition, as expected the *E*-isomer **1** corresponding to the natural all-*E* OS was slightly more active than the *Z*-isomer **2** on both cyclases.

Next, compound **3** designed to be cyclase-activated so as to mimic the tetracyclic C-20 protosteryl or dammarenyl cations, and the corresponding *N*-oxide **4**, were assayed on both cyclases. The purpose of assaying *N*-oxide **4** was based on the proposal that, generally, the amine *N*-oxide group has been shown to be able to

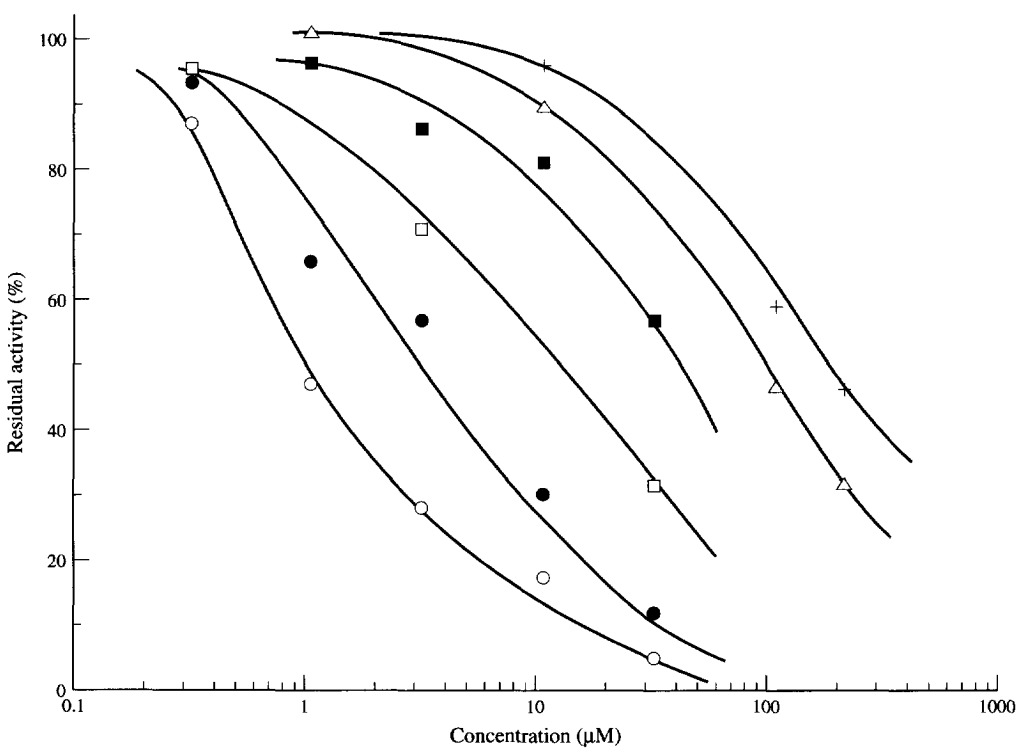

Fig. 2. Typical OSCC and OSβAC inhibition curves obtained in the presence of various inhibitors. Inhibition of OSCC by **6** (●) and **3** (■); inhibition of OSβAC by **6** (○), **3** (□), **1** (△) and **2** (+). The activity of the control (without inhibitor) was normalized to 100%.

mimic positively charged intermediates by charge–charge or dipole–dipole interaction [13]. Compounds **3** and **4** were moderately active on both cyclases. In addition, **5** lacking the 2,3-epoxy function showed an inhibitory activity on OS cyclases from maize and peas which was very similar to that found for **3** or **4**.

We then assayed the inhibition caused by **6** because it could possibly interfere with the negative charge delivered by the OSβAC in order to stabilize the lupenyl cation involved during β-amyrin cyclization, but not during cycloartenol cyclization (Scheme 1) and thus could be more selective for OSβAC than OSCC. The data (Table 1) show that compound **6** was an effective inhibitor of both OSCC and OSβAC. The I_{50} values of **6** (1–3 μM) compared favourably with the apparent K_m (125 μM) for both plant cyclases. Moreover, the affinity of **6** is one of the highest among the compounds known to inhibit plant OS-cyclases [16–19].

The I_{50} values determined for maize OSCC and pea cotyledons OSβAC indicate that the compounds tested generally displayed a similar activity on both cyclases. However, the most effective inhibitors, compounds **3** and **6**, tend to be more effective on OSβAC than on OSCC (Fig. 2). The selectivity of the most effective inhibitors was also estimated by the ratio of $\beta(\alpha)$-amyrin over cycloartenol produced in the same microsomal preparation of pea embryos incubated in the presence of any of these compounds at a concentration of 30 or 100 μM, depending on their affinity (Table 1). This ratio which was normally in the range of 2.0 in the absence of inhibitor, did not show major variation in the presence of inhibitors, except for **6**, confirming that these compounds were equally active on both plant cyclases. However, it is interesting to note that this ratio was lowered to 1.65 in the case of **6**, indicating a more effective inhibition of OSβAC than of OSCC in this preparation.

The absence of selectivity of **1** between OSβAC and OSCC is in sharp contrast with the observed high selectivity previously observed for the corresponding cyclic compound **7** which was shown to be totally selective for OSCC [16, 19]. It is noteworthy to mention that such a lack of selectivity has been also found for another acyclic azasqualene inhibitor, 2-aza-2,3-dihydrosqualene, for which the acyclic form is active [19].

The results of the present study show that the measured affinities for compounds **1**, **3** and **6** are spread over two orders of magnitude in the sequence **6** $\gg$ **3** > **1**. This sequence shows that the more external the nitrogen atom within the squalene framework, the more potent is the inhibition. Moreover, the efficiency of the inhibition also parallels the distance between the oxirane and the nitrogen atom, suggesting that these acyclic derivatives might better accommodate the conformations of late HEIs within the cyclization process, i.e. HEIs possessing a carbenium ion at *pro*-C-20 or *pro*-C-25. It is noticeable that the relative efficiency of **1** and **3** was reverse to that found for the corresponding rigid cyclic HEI analogues [19, 21].

The activity of **1** and **2** on both plant cyclases was much weaker than that found in the case of mammalian and fungal OS-lanosterol cyclases (OSLC) [22]. In contrast, **6** has an efficiency comparable to that of the most powerful known inhibitors of these cyclases [18, 19]. The potent inhibition of OSβAC by **6** could result from a partial cyclization of **6** to produce *in situ* a structure able to mimic efficiently the lupenyl cation involved in the cyclization process of β-amyrin. However, the fact that **6** is similarly active on OSCC, which does not involve such a HEI, would better suggest that **6** acts as a mimic of the first transient HEI at C-2, resulting from the oxirane cleavage, which is common to both cyclases.

The affinity of 19-aza-oxidosqualene (**3**) for OSβAC contrasted with the failure of 20-aza-dammaran-3β-ol, a cyclized aza analogue of the *pro*-C-20 HEI, to inhibit pea OSβAC [21]. This difference could reflect the ability of **3** to be cyclase-activated to generate slowly *in situ* the suitable C-20 HEI analogue involved in the reaction pathway of both OSβAC and OSCC. Thus, possible time dependence of the inhibition of maize OSCC by **3** was examined, first indirectly, by measuring the time-dependent formation of cycloartenol in the absence of inhibitor and in the presence of a low concentration of **3**. The 8-azadecalin (**7**) was used as a control for a non-activated inhibitor of the cyclase [19]. However, no deviation from linearity could be observed when **3** or **7** were added to the reaction medium (data not shown), indicating the steady-state inhibition of OSCC by **3** and **7** during a period where OSCC product formation is constant.

The time-dependence of the inhibition by **3** was next assessed more directly by preincubating **3** with OSCC. At intervals, the OSCC activity was assayed. No significant loss of activity was observed following a series of preincubation times with **3**, in good accordance with the preceding experiment (data not shown).

These data would suggest either that the acyclic form of **3** was the active species or that possible cyclization of **3** would be much slower than that of OS and thus not detectable under our experimental conditions. The fact that a similar activity was obtained with **5** (Table 1), which otherwise cannot undergo cyclization because of the lack of the epoxy function, would better support the first possibility. This result is in contrast with recent findings indicating that **3** is able to inactivate OSLC from pig liver in a time-dependent manner [26]. This absence of activation of **3** by the plant OSCC could be due particularly to its lower affinity for the plant cyclases in comparison to that measured for the mammalian OSLC [24] (I_{50} = 1.5 μM) or to a low degree of homology between these cyclases from distinct organisms.

Taken together, the present data obtained with plant cyclases and that previously obtained with other organisms show that going from C-2 (2-aza-2,3-dihydrosqualene and **6**), *pro*-C-8 (i.e. **1**) and *pro*-C-20 (i.e. **3**) HEIs, a progressive specificity was acquired. Indeed, 2-aza-2,3-dihydrosqualene [18] and **6** [25 and this work] inhibited all types of OSC, **1** was active on yeast

and animal OSC [22, 23] and finally **3** was specific towards animal OSC [24]. The difference in efficiency of **1** and **6** between the plant, the mammalian or fungal OS cyclases [22, 24, 26] could reflect the fact that the enzymic domains complementary to the *pro*-C-8 and *pro*-C-20 HEIs are structurally different in these distinct cyclases. Indeed, recent protein and nucleotide sequences indicate that OS-cyclases from different organisms show a relatively low degree of homology [27–30].

Finally, the present work has confirmed the validity of our previous approach in designing new inhibitors of plant sterol cyclases based on the HEI analogue concept. Whatever the detailed mechanism of action of **3** and **6** may be, these compounds can be considered as new inhibitors of both OSCC and OSβAC. They are useful tools for studying the mechanism of action of these fascinating enzymes, besides having potential use in controlling sterol biosynthesis.

EXPERIMENTAL

Instrumentation. Radioactivity was determined with a Packard XL-6742 scintillation counter which was calibrated for correction to decompositions per minute (dpm).

Labelled compounds. [3-^{3}H]-2,3-(*RS*)-Oxidosqualene was synthesized as described before [31].

Inhibitors. Compounds **1–4**, **6** and **7** were obtained as described before [22, 24, 25, 16].

2,3-*Oxidosqualene-cyclase assay.* Microsomes from maize seedlings, pea cotyledons and pea embryos were prepd as previously described [16, 18]. [^{3}H]-2,3-(*RS*)-Oxidosqualene (100 μM final, 2.5×10^5 dpm) emulsified in T80 (1 mg ml^{-1} final concn) was added to microsomes (1 ml = 3–6 mg protein) and the reaction mixt. was incubated at 30° with gentle stirring for 1.5 hr during which time the reaction proceeded linearly. The reaction was stopped by addition of 6% (w/v) ethanolic KOH (1 ml). A boiled enzyme prepn was incubated in parallel. The neutral lipids were extracted with hexane and analysed by TLC on silica gel using CH_2Cl_2 (×2) as solvent. The 4,4-dimethylsterols and amyrins (R_f 0.45) were sepd from the 4α-methylsterols (R_f 0.40) and the 4-desmethylsterols (R_f 0.30) and eluted from the gel after scanning the plate for radioactivity. In the case of production of both cycloartenol and β-amyrin, i.e. with pea embryo microsomes, the 4,4-dimethylsterol and amyrin fr. was subjected to acetylation under standard conditions [32] after addition of authentic $\alpha + \beta$ amyrin (300 μg) and cycloartenol (300 μg) as carrier. After freeze-drying of reactants, this fr. was subjected to analyt. argentation TLC (10% $AgNO_3$) in which cyclohexane–toluene (7:3) was the developing solvent (2 runs). After migration, amyrin-acetates (R_f 0.65) were readily sepd from cycloartenyl acetate (R_f 0.49) and 24-methylenecycloartanol (R_f 0.34), which were identified by co-migration with authentic standards. The different frs were scraped off the plate and associated radioactivity detemined by liquid scintillation counting.

Determination of inhibition constants. The different microsomal cyclases were assayed as described above in the presence of a range of concns of the inhibitors to be tested and the I_{50} values determined (I_{50} *corresponds to the inhibitor concn which reduces the observed reaction rate by* 50%). The inhibitors were added as an emulsion with OS and Tween-80 without any preincubation time.

Preincubation in the presence of **3** *and maize OSCC.* Compound **3** (5 μM, final concn) emulsified in Tween-80 (1 mg ml^{-1} final concn) was added to maize seedling microsomes (1 ml = 3–6 mg protein) and the reaction mixt. was incubated at 30°. After various times of incubation, OSCC was assayed as described above. In control experiment, microsomes were preincubated in the presence of Tween-80 alone.

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