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# COMPARATIVE STUDY OF THE INHIBITION OF STEROL BIOSYNTHESIS IN *RUBUS FRUTICOSUS* SUSPENSION CULTURES AND *ZEA MAYS* SEEDLINGS BY *N*-(1,5,9-TRIMETHYLDECYL)-4α,10-DIMETHYL-8-AZA-*TRANS*-DECAL-3β-OL AND DERIVATIVES

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**Key Word Index**—*Rubus fruticosus*; Rosaceae; *Zea mays*; Poaceae; *N*-(1,5,9-trimethyldecyl)-4α,10-dimethyl-8-aza-*trans*-decal-3β-ol; sterol biosynthesis inhibitor; 9β,19-cyclopropyl sterols; $\Delta^8$-sterols; $\Delta^{8,14}$-sterols.

**Abstract**—The nitrogen substituents present in tridemorph and fenpropimorph, which are systemic fungicides, have been linked to an 8-aza-bicyclic skeleton leading to *N*-(1,5,9-trimethyldecyl)-4α,10-dimethyl-8-aza-*trans*-decal-3β-ol and *N*-(3-(4-tert-butyl phenyl-)2-methyl)-propyl-8-aza-4α,10-dimethyl-*trans*-decal-3β-ol respectively. The latter two compounds present in a stable molecule key structural elements of unstable C-8 and C-9 carbocationic high-energy intermediates which occur during the reactions catalysed by the $\Delta^8 \rightarrow \Delta^7$-sterol isomerase and the cycloeucalenol–obtusifoliol isomerase, respectively. When given to either bramble cell suspension cultures or maize seedlings, they led to a spectacular accumulation of 9β,19-cyclopropyl sterols and were in that respect much more efficient than any known molecules and in particular than the *N*-benzyl decalin previously described which led to accumulation of $\Delta^8$-sterols. Surprisingly, treatment of the plant cells by the *N*-oxide derivatives of the *N*-benzyl decalin resulted in dramatic accumulation of $\Delta^{8,14}$-sterols.

## INTRODUCTION

In recent work [1], *N*-benzyl-8-aza-4α,10-dimethyl-*trans*-decal-3β-ol (**1**) was reported to be a potent inhibitor of the cycloeucalenol–obtufoliol isomerase (COI) and the $\Delta^8 \rightarrow \Delta^7$-sterol isomerase and it is the first rationally designed inhibitor of these two enzymes. Although this molecule was mainly assayed on the isolated enzymes, preliminary data gave evidence that it also works *in vivo* and inhibits both enzymes in suspension cultures of bramble cells [1]. The present work extends this observation in testing the azadecalins in whole plants (maize seedlings) in addition to bramble cell cultures. Moreover, new azadecalins are described; in particular, *N*-(1,5,9-trimethyldecyl)-4α,10-dimethyl-8-aza-*trans*-decal-3β-ol (**2**), an azadecalin possessing, in addition to the 3β-OH bicyclic skeleton, a steroid-like side chain, was shown to be remarkably active and to act at a much lower concentration than previously described molecules inhibiting the same enzymes. Our attention was also given to the *N*-oxide derivatives of 8-azadecalins. Surprisingly, these have been shown to act on other enzymatic targets than the parent amines. Careful examination of the sterol profiles of the bramble cells and maize seedlings grown in the presence of *N*-oxide derivatives led to the conclusion that the main enzymatic target of these latter compounds is the $\Delta^{8,14}$-sterol $\Delta^{14}$-reductase.

## RESULTS

### *Description of the molecules used as inhibitors*

The *N*-benzyl decalin (**1**) has been described previously [1]. Previous data obtained in bramble cells and preliminary results on maize seedlings have shown that **1**, although very active on the isolated enzymatic targets (COI and $\Delta^8 \rightarrow \Delta^7$-sterol isomerase) is less active *in vivo* than expected. In order to increase the *in vivo* activity, the nitrogen substituents present in tridemorph (**3**) and fenpropimorph (**4**), which are systemic fungicides translocated efficiently in plants [2, 3] and potent sterol biosynthesis inhibitors in both fungi and plants [4–7], were linked to the 8-aza-bicyclic skeleton leading to **2** and **5**. As shown in Fig. 1, it appears that **2** mimics closely the cationic high-energy intermediates (HEI) **6** and **7** involved in the catalytic pathway of the COI and the $\Delta^8 \rightarrow \Delta^7$-sterol isomerase, since the stable molecule **2** possesses electronic, electrostatic (the positive charge at C-8) and structural (the bicyclic skeleton and the quasi-sterol side chain) elements of the unstable HEI. The activity of **2** has been compared to that of **10**, an 8-azadecalin possessing a linear $n$-$C_{12}H_{25}$ chain. An improved translocation could also result from the N-oxidation. Indeed, the *N*-oxide derivatives of tertiary amines have a much higher water solubility than the parent amines. In addition,

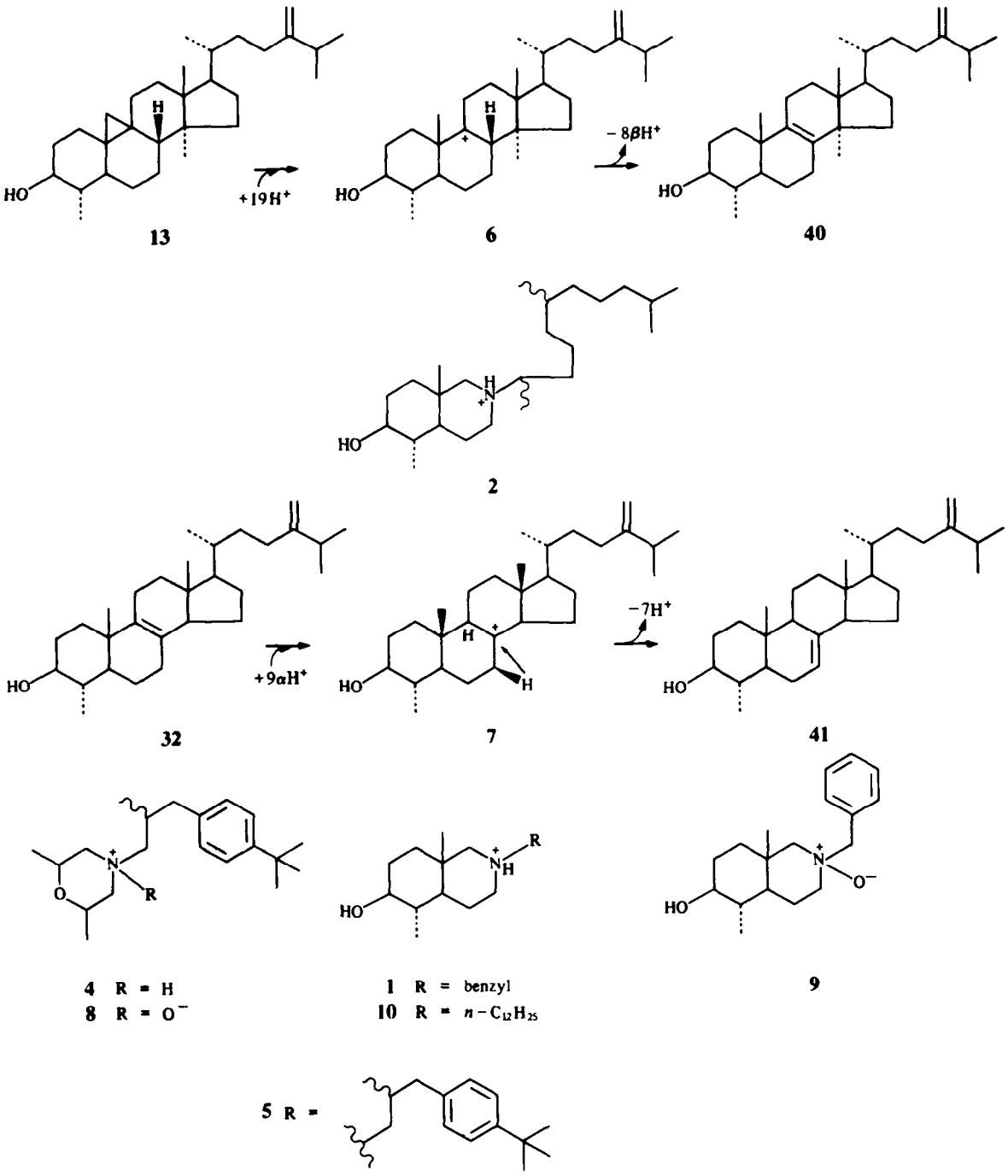


Fig. 1. Hypothetical reaction pathways for cycloeucalenol–obtusifoliol isomerase (13 → 6 → 40) and $\Delta^8 \rightarrow \Delta^7$-sterol isomerase (32 → 7 → 41) showing the analogies existing between 8-azadecalins such as 2 and the carbocationic high-energy intermediates 6 and 7.

though possessing a strong dipolar moment [8], they are globally neutral over a wide range of pH [8]. In order to assess this point, the *N*-oxide derivative of fenpropimorph (8) (an already available compound) [9] and of the *N*-benzyl-8-azadecalin (9) (a new compound synthesized by us) were assayed with our biological materials. The newly synthesized compounds (1, 2, 5, 9 and 10) and the already described molecules (3, 4 and 8) were tested on both suspension cultures of bramble cells and maize seedlings.

*Effect of the inhibitors on suspension cultures of bramble cells*

The various inhibitors were given to the cells at several concentrations (0.5–20 mg/l). In general, they display relatively low toxicity. However, a significant decrease in growth was observed for concentrations higher than 10 mg/l. In some cases (2 and 3), complete growth inhibition was obtained for concentrations close to

20 mg/l. We shall detail here only the results obtained with low (1 mg/l.) amounts of the chemicals. These results were the most significant since at this concentration of inhibitor an identical dry weight of cells was obtained at the end of the culture, allowing comparison of the effects on sterol biosynthesis. Profound changes in sterol profile resulted following treatment with **1**, **2**, **4**, **5**, **9** and **10** (Table 1). The following clearcut differences were observed: (1) the 8-azadecalins (**2**, **5** and **10**) led to an enormous accumulation of cyclopropyl sterols; amongst them cycloeucalenol (**13**), 24-methylenepollinastanol (**15**) and 24-methylpollinastanol (16) were the major components; (ii) fenpropimorph (**4**) and *N*-benzyl-8-azadecalin (**1**) treatment resulted in a strong accumulation of the $\Delta^8$-sterols (**25** and **26**); and (iii) *N*-benzyl-8-azadecalin-*N*-oxide (**9**) and **8** (data not shown) resulted in dramatic accumulation of $\Delta^{8,14}$-sterols and amongst them **21** and **22** predominated. A new picture of the situation emerges when the 4-desmethylsterol fraction is considered (Table 2A): (i) only **2** and **5** led to a strong accumulation of cyclopropylsterols; no traces of $\Delta^8$- and $\Delta^{8,14}$-sterols were detected even in the case of **2**; (ii) **1** and fenpropimorph (**4**) resulted specifically in the accumulation of $\Delta^8$-sterols; and (iii) the *N*-oxide derivatives of **1** and **4** (**9** and **8** respectively) led to $\Delta^{8,14}$-sterol accumulation. In the cases of **1**, **4**, **8** and **9**, cyclopropyl sterols were no longer detectable. A complete array of results was also obtained with cells treated with inhibitors at 5 mg/l. The results are essentially identical to those shown above but the general trend was that the relative amount of cyclopropyl sterol increased strongly in all cases except that of **9**.

*Effects of the inhibitors on maize seedlings*

The maize seedlings were watered daily with an aqueous solution of the various inhibitors for 15 days. Two concentrations (1 and 5 mg/l.) of inhibitors were used. This treatment led to some growth inhibition. The

Table 1. Sterol content of suspension cultures of bramble cells grown in the presence of various sterol biosynthesis inhibitors (**1**, **2**, **4**, **5**, **8**, **9** and **10**) (1 mg/l.). The cells were grown for 4 weeks, then harvested. The dry weight was 2.1 ± 0.3 g

| | **4** | **5** | **1** | **9** | **10** | **2** | Control |
|---|---|---|---|---|---|---|---|
| | | | (% of total sterols) | | | | |
| TRITERPENES | | | | | | | |
| α- and β-amyrin, unidentified triterpenes | 4.30 | 5.04 | 10.56 | 5.16 | 7.96 | 11.41 | 2 |
| Cycloartenol (**11**) | 2.30 | 3.52 | 6.30 | 2.91 | 3.97 | 5.88 | 1 |
| 24-Methylenecycloartanol (**12**) | 1.48 | 11.03 | 3.54 | 1.82 | 9.17 | 24.00 | 0.5 |
| 4α-METHYL STEROLS | | | | | | | |
| Cycloeucalenol (**13**) | 27.16 | 45.79 | 12.19 | 7.17 | 38.03 | 32.53 | tr |
| Cyclofontumienol (**14**) | tr | 2.28 | 3.37 | —* | 1.06 | 1.12 | — |
| 31-Norcyclobranol (**27**) | — | 1.39 | — | — | — | — | — |
| 4α-Methyl-5α-ergosta-8,14,24(28)-trien-3β-ol (**30**) | — | — | — | 5.40 | — | — | — |
| 4α-Methyl-5α-stigmasta-8,14,*Z*-24(28)-trien-3β-ol (**31**) | — | — | — | 9.82 | — | — | — |
| 4α-Methyl-5α-ergosta-8,24(28)-dien-3β-ol (**32**) | — | — | — | 0.69 | — | — | — |
| 4α-Methyl-5α-stigmasta-8,*Z*-24(28)-dien-3β-ol (**33**) | — | — | — | 3.57 | — | — | — |
| 24-Methylenelophenol (**41**) | — | — | — | — | — | — | 0.1 |
| 4-DESMETHYL STEROLS | | | | | | | |
| 24-Methylenepollinastanol (**15**) | 4 | 12.72 | — | — | 10.31 | 12.51 | — |
| 24-Methylpollinastanol (**16**) | tr | 4.02 | — | — | — | 5.68 | — |
| 24-Ethylidenepollinastanol (**17**) | — | 2.55 | — | — | 1.46 | 2.24 | — |
| 24-Ethylpollinastanol (**18**) | — | — | — | — | — | 0.53 | — |
| 5α-Ergosta-8,14,24(28)-trien-3β-ol (**19**) | — | — | — | — | — | — | — |
| (24ξ)-24-Methyl-5α-cholesta-8,14-dien-3β-ol (**20**) | — | 0.57 | — | — | — | — | — |
| 5α-Stigmasta-8,14,*Z*-24(28)-trien-3β-ol (**21**) | 2.28 | — | — | 15.04 | — | — | — |
| (24*R*)-24-ethyl-5α-cholesta-8,14-dien-3β-ol (**22**) | — | 2.06 | — | 15.46 | — | — | — |
| Fecosterol (**23**) | 1.21 | 0.57 | — | — | — | — | — |
| (24ξ)-24-Methyl-5α-cholest-8-en-3β-ol (**24**) | 0.90 | 0.57 | — | — | — | — | — |
| 5α-Stigmasta-8,*Z*-24(28)-dien-3β-ol (**25**) | 15.36 | 1.37 | 28.21 | 15.04 | 1.90 | — | — |
| (24*R*)-24-ethyl-5α-cholest-8-en-3β-ol (**26**) | 37.37 | 0.80 | 20.06 | 8.78 | — | — | — |
| 24-Methylene cholesterol (**27**) | 0.42 | 0.57 | — | — | 1.84 | — | — |
| Campesterol (**28**) | 0.60 | 0.57 | — | — | 2.63 | — | 14 |
| Isofucosterol (**38**) | — | 1.14 | 8.33 | — | 11.64 | 1.08 | 12 |
| Sitosterol (**29**) | 4.54 | 1.15 | 7.44 | 9.14 | 10.02 | 3.02 | 70 |
| Total cyclopropyl sterols | 35.25 | 83.30 | 25.40 | 11.90 | 64.01 | 84.09 | — |
| Total $\Delta^{8,14}$-sterols | — | 4.91 | — | 45.72 | — | — | — |
| Total $\Delta^8$-sterols | 54.85 | 3.31 | 48.27 | 28.08 | 1.90 | — | — |
| Total $\Delta^5$-sterols | 5.56 | 3.44 | 15.78 | 9.14 | 26.13 | 4.10 | — |
| Total non-sterolic triterpenoids | 4.33 | 5.04 | 10.56 | 5.16 | 7.96 | 11.41 | — |
| Total weight of sterols (mg/g dry wt) | 7.50 | 8.0 | 5.70 | 4.75 | 3.4 | 4.5 | 3.4 |

* Not detectable.

Table 2. Relative proportion of the different categories of sterols in (A) the 4-desmethylsterol fraction and (B) the total sterol fraction from bramble cells suspension cultures grown in the presence of **1**, **2**, **4**, **5**, **9** and **10** (1 mg/l.)

| | | Control | **1** | **9** | **10** | **2** | **5** | **4** |
|---|---|---|---|---|---|---|---|---|
| | | (% of total sterols) | | | | | | |
| (A) | Total cyclopropyl sterols | 0 | 0 | 0 | 30 | 84 | 63 | 1 |
| | Total $\Delta^{8,14}$-sterols | 0 | 0 | 48 | 0 | 0 | 16 | tr |
| | Total $\Delta^{8}$-sterols | 0 | 75 | 38 | 5 | tr | 10 | 90 |
| | Total $\Delta^{5}$-sterols | 100 | 25 | 14 | 65 | 16 | 12 | 9 |
| (B) | Total cyclopropyl sterols | 1 | 25 | 12 | 64 | 85 | 84 | 35 |
| | Total $\Delta^{8,14}$-sterols | 0 | 0 | 46 | 0 | 0 | 5 | tr |
| | Total $\Delta^{8}$-sterols | 0 | 48 | 28 | 2 | tr | 3 | 55 |
| | Total $\Delta^{5}$-sterols | 96 | 16 | 9 | 26 | 4 | 3 | 4 |

strongest growth inhibition ($45 \pm 7\%$ with respect to control seedlings) was obtained with fenpropimorph (**4**) (5 mg/l.) After 10 days of growth in the presence of **4**, necrotic spots were detectable. The 8-azadecalins (**2** and **5**) at identical concentrations led to less growth inhibition ($23 \pm 5\%$ with respect to control seedlings) and necrotic spots were not observed. The sterol profile of the roots from maize seedlings is given in Table 3. The results obtained paralleled those obtained with bramble cells. However, some differences were apparent. Two groups of compounds arose: (i) fenpropimorph (**4**) and the decalins **2** and **5** led to an almost complete replacement of $\Delta^5$-sterols by cyclopropyl sterols; amongst them cycloeucalenol (**13**), dihydrocycloeucalenol (**34**), and 24-methylpollinastanol (**16**) were the major components; (ii) the *N*-benzyl decalin (**1**) was much less efficient than the three compounds mentioned above and led to a mixture of cyclopropyl- and $\Delta^8$-sterols; (iii) the *N*-benzyl decalin *N*-oxide (**9**) led to a strong accumulation of $\Delta^{8,14}$-sterol (**20**, **22** and **35**). A second set of data was obtained with maize seedlings watered with a 1 mg/l. solution of inhibitors (**1**, **2** and **4**). The results (data not shown) confirm that **2** and **4** cause a strong accumulation of cyclopropyl sterols (70% for both compounds) whereas **1** produced a moderate accumulation of $\Delta^8$-sterols. All the sterols isolated from treated bramble cells and maize seedlings were identified by their $^1$H NMR and mass spectra, as described previously [1, 7]. A thorough study of the C-24 configuration of the 24-methyl of 9$\beta$,19-cyclopropyl sterols (**16** and **34**) has been described elsewhere [10]. This study has shown that **16** and **34** are mixtures of epimers at C-24, containing 85 and 55% of the (24*S*)-24-methyl epimer, respectively, and 15 and 45% of the (24*R*)-24-methyl epimer, respectively.

## DISCUSSION

From the data obtained in the present work, the following conclusions emerge: (i) the 8-azadecalins are potent sterol biosynthesis inhibitors and inhibit three enzymes—the COI, the $\Delta^8 \rightarrow \Delta^7$-sterol isomerase and the $\Delta^{8,14}$-sterol reductase; (ii) the effects on bramble cell suspension cultures and on maize seedlings are essentially identical; (iii) *N*-(1,5,9-trimethyldecyl)-8-aza-4$\alpha$,10-dimethyl-*trans*-decal-3$\beta$-ol (**2**) is a remarkably potent sterol biosynthesis inhibitor in plants; its target enzyme is principally the COI; (iv) the *N*-oxide derivative of *N*-benzyl-8-azadecalin selectively inhibits the $\Delta^{8,14}$-sterol reductace.

*The 8-azadecalins are potent sterol biosynthesis inhibitors.* As shown in Tables 1 and 3, these molecules when given to either bramble cell suspension cultures or maize seedlings lead to a strong decrease in $\Delta^5$-sterols. In the case of bramble cells treated with **2** and **5** (1 mg/l.), residual $\Delta^5$-sterols are less than 4% of total sterols. Amounts of $\Delta^5$-sterols lower than 4% are never obtained even when concentrations of sterol biosynthesis inhibitors higher than 1 mg/l. are used. This observation can be explained taking into account that the bramble cells present at the beginning of the culture are about 10% of the cells present at the end of the culture and that the turnover of sterols is very low [11]. Therefore we believe that most on the $\Delta^5$-sterols present at the end of the cultures grown in the presence of inhibitors originate from those present at the beginning of the experiment. When an aliquot fraction (10% of the cells treated with **2** for 4 weeks and possessing the sterol profile shown in Table 1) was transferred to a fresh culture medium containing **2** again and the cells were allowed to grow until the stationary phase, the residual $\Delta^5$-sterol content was shown to decrease markedly. After five transfers on a culture medium containing **2**, the $\Delta^5$-sterols disappeared almost completely ($< 0.2\%$) and were replaced by cyclopropyl sterols (95%), supporting the assumption that the cells initially containing $\Delta^5$-sterols were diluted through the successive transfers (Fig. 2).

*The target enzymes of the 8-azadecalins were the COI, the $\Delta^8 \rightarrow \Delta^7$-sterol isomerase and the $\Delta^{8,14}$-sterol reductase.* The results presented in Tables 1–3 show clearly that 8-azadecalins lead to essentially similar effects in bramble cell suspension cultures and maize seedlings. Whereas **2**, **5** and **10** lead to a strong accumulation of cyclopropyl sterols, **1** leads to an accumulation of mostly $\Delta^8$-sterols and **9** of $\Delta^{8,14}$-sterols. These results suggest that one major target enzyme of **2**, **5** and **10** is the COI; the major target enzyme of **1** would be the $\Delta^8 \rightarrow \Delta^7$-sterol isomerase and for **9** it would be the $\Delta^{8,14}$-sterol reductase. However, caution is required with *in vivo* results: the accumulation of intermediates, e.g. cyclopropyl sterols, gives reasonable evidence that the COI is inhibited, but does not exclude other targets situated downstream of the COI in the biosynthetic pathway. Indeed, if the biosynthetic flow is completely stopped after the COI, inhibition of the other enzymes could not become apparent. To clarify this question, direct action of the inhibitor on the isolated presumed target enzyme would be necessary. It was shown previously [1, 9, 12] that **1** and **4** inhibited strongly

Table 3. Sterol content of roots from maize seedlings grown in the presence of the sterol biosynthesis inhibitors (**1**, **2**, **4**, **5**, **9**). The concentration of inhibitor used was 5 mg/l. except for **5** (3.5 mg/l.). The seedlings were grown for 15 days and then harvested

| | **4** | **5*** | **1** | **9** | **2** | Control |
|---|---|---|---|---|---|---|
| | (% of total sterols) | | | | | |
| TRITERPENES | | | | | | |
| α- and β-amyrin | 0.71 | 1.13 | 0.61 | 0.23 | 0.35 | tr |
| Cycloartenol (**11**) + cycloartanol (**39**) | 3.82 | 8.26 | 5.67 | 2.02 | 1.58 | — |
| 24-Methylenecycloartenol (**12**) | 2.18 | 5.87 | 0.95 | 0.18 | 1.53 | tr |
| Other triterpenes | 0.39 | 0.86 | 0.13 | 0.02 | 0.30 | tr |
| 4α-METHYL STEROLS | | | | | | |
| Cycloeucalenol (**13**) | 30.08 | 33.43 | 9.61 | 3.22 | 32.91 | 1 |
| Cyclofontumienol (**14**) | 2.55 | 1.61 | 0.66 | 0.10 | 2.55 | — |
| 31-Norcyclobranol (**27**) | 3.17 | — | 1.18 | — | 6.52 | — |
| 24-Dihydrocycloeucalenol (**34**) | 15.36 | 8.64 | 3.95 | 0.98 | 14.53 | — |
| 4α-Methyl-5α-ergosta-8,14,24(28)-trien-3β-ol (**30**) | — | — | — | 2.67 | — | — |
| Obtusifoliol (**40**) | — | — | tr | tr | — | 1 |
| 4-DESMETHYL STEROLS | | | | | | |
| 24-Methylenepollinastanol (**15**) | 5.70 | — | — | — | 3.58 | — |
| 24-Methylpollinastanol (**16**) | 30.69 | 23.37 | 11.37 | — | 30.70 | — |
| 24-Ethylidenepollinastanol (**17**) | 0.88 | 0.53 | — | — | — | — |
| 24-Ethylpollinastanol (**18**) | 0.74 | 0.52 | — | — | 0.70 | — |
| (24ξ)-24-Methyl-5α-cholesta-8,14-dien-3β-ol (**20**) | — | 0.91 | — | 10.83 | — | — |
| (24ξ)-24-Ethyl-5α-cholesta-8,14,*E*-22-trien-3β-ol (**35**) | — | 1.79 | — | 11.17 | — | — |
| (24*R*)-24-Ethyl-5α-cholesta-8,14-dien-3β-ol (**22**) | — | 0.70 | — | 14.39 | — | — |
| Fecosterol (**23**) | — | — | — | — | — | — |
| (24ξ)-24-Methyl-5α-cholest-8-en-2β-ol (**24**) | 1.37 | — | 4.57 | — | — | — |
| (24ξ)-24-Ethyl-5α-cholesta-8, *E*-22-dien-3β-ol (**36**) | — | — | 11.65 | — | — | — |
| (24*R*)-24-Ethyl-5α-cholest-8-en-3β-ol (**26**) | — | — | 2.68 | — | — | — |
| 24-Methylenecholesterol (**27**) | — | — | — | — | — | 1 |
| Campesterol (**28**) | 0.44 | 2.45 | 12.56 | 15.28 | 0.76 | 23 |
| Stigmasterol (**37**) | 1.90 | 7.04 | 25.08 | 30.25 | 3.51 | 46 |
| Sitosterol (**29**) | 0.10 | 0.83 | 7.83 | 8.65 | 0.47 | 22 |
| Isofucosterol (**38**) | — | — | tr | — | — | 2 |
| Total cyclopropyl sterols | 95.57 | 83.30 | 33.52 | 6.53 | 94.90 | 2 |
| Total $\Delta^{8,14}$-sterols | — | 3.40 | — | 36.39 | — | — |
| Total $\Delta^{8}$-sterols | 1.37 | 1.73 | 19.23 | — | — | — |
| Total $\Delta^{5}$-sterols | 2.44 | 10.31 | 45.47 | 54.18 | 4.75 | 96 |
| Total weight of sterols (mg/g dry wt) | 1.90 | 2.30 | 5.00 | 6.00 | 2.35 | 2.5 |

microsomal COI and $\Delta^8 \rightarrow \Delta^7$-sterol isomerase from maize embryos. In the same cell-free system, **2** and **5** were shown to be also potent inhibitors of both the COI and $\Delta^8 \rightarrow \Delta^7$-sterol isomerase [22]. Therefore, the selectivity observed *in vivo* in the cases of **2** and **5** could reflect the following situation: when the COI is totally inhibited, the inhibition of potential target enzymes (e.g. the $\Delta^8 \rightarrow \Delta^7$-sterol isomerase and $\Delta^{8,14}$-sterol reductase) situated downstream of the COI is no longer observable. In contrast, the accumulation of $\Delta^8$-sterols in the case of **1** and of $\Delta^{8,14}$-sterols in the case of **9** is a good indication that at least *in vivo* **1** and **9** inhibit rather selectively the $\Delta^8 \rightarrow \Delta^7$-sterol isomerase and the $\Delta^{8,14}$-sterol reductase, respectively. Two important conclusions should be pointed out. Firstly, compound **2** is the most potent inhibitor of the COI known at this time: at a concentration of 1 mg/l., it led to replacement of almost all $\Delta^5$-sterols by cyclopropyl sterols (essentially cycloeucalenol, 24-methylenepollinastanol and 24-methylpollinastanol) in bramble cells. At a concentration of 0.1 mg/l., it is still very active (data not shown) and led to accumulation of more than 50% of cyclopropyl sterols. The fact that in these conditions of incomplete inhibition no trace of $\Delta^8$-sterols is detectable suggests that **2** displays a very high selectivity towards the COI, at least *in vivo*. As discussed above, when the bramble cells grown on **2** (1 mg/l.) were transferred onto a new culture medium containing **2** (1 mg/l.), the residual $\Delta^5$-sterols disappeared almost completely (Fig. 2). Surprisingly after seven transfers of this type, the growth of these cells was not significantly different from that of control cells. These findings show clearly that the replacement of nearly 100% of the $\Delta^5$-sterols present normally in bramble cells by 9β,19-cyclopropyl sterols does not affect the growth of the cells. This conclusion raises the important problem of the location of cyclopropyl sterols in bramble cells treated with **2**. Recent data

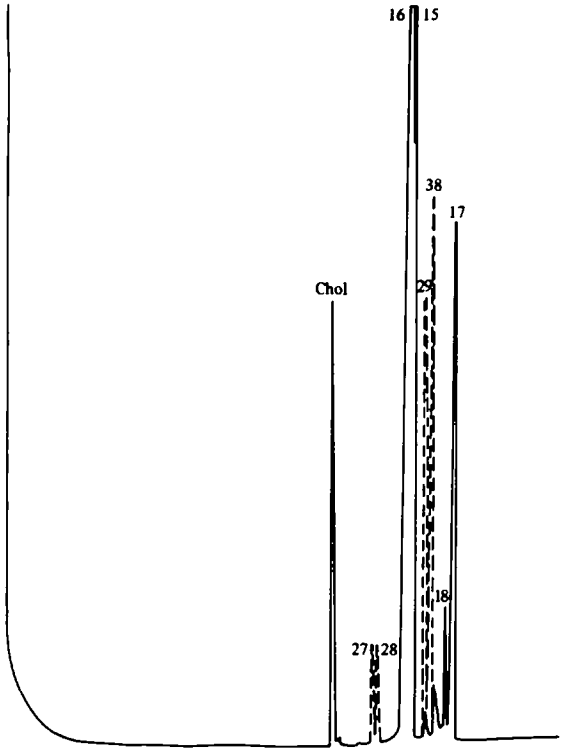


Fig. 2. 4-Desmethylsterol profile determined by capillary GC of bramble cell suspension cultures. (- - -) Sterol profile of control cells; (——) sterol profile of cells after five transfers on a culture medium containing **2** (1 mg/l.). **15**, **16**, **17**, **18**: 24-methylenepollinastanol, 24-methylpollinastanol, 24-ethylidenepollinastanol, 24-ethylpollinastanol present, respectively, in bramble cells treated with **2**. **27**, **28**, **29** and **38**: 24-methylenecholesterol, 24-methylcholesterol, sitosterol and isofucosterol present, respectively, in control bramble cells. Chol: external standard of cholesterol. All compounds listed above are acetates except cholesterol.

show without ambiguity that these sterols are distributed in pericellular and intracellular membranes (mitochondria, endoplasmic reticulum, plasma membrane) [14, 15]. This result raised the following question: What are the consequences of the presence of cyclopropyl sterols on membrane structure and function? To answer this question, a careful analysis of phospho- and glycolipids and a study of the effect of the changes in the sterol profile on various membrane-bound enzymatic activities are in progress in our laboratory. Compound **2** has been shown to inhibit also the 2,3-oxidosqualene–cycloartenol (lanosterol) cyclase in cell-free extracts of maize embryos, pea seedlings, bramble cell suspension cultures and rat liver, but at concentrations higher than those necessary for the inhibition of the COI [13].

The second point is that the major target enzyme of **9** is the $\Delta^{8,14}$-sterol reductase. This is an unexpected result, which shows that the replacement of a tertiary amine by its *N*-oxide derivative leads to a profound change in its inhibitory properties. Whereas the tertiary amines **1** and **4** are inhibitors of the $\Delta^8 \rightarrow \Delta^7$-sterol isomerase and of the COI, their *N*-oxide derivatives, **9** and **8**, respectively, appear to be selective inhibitors of the $\Delta^{8,14}$-sterol reductase *in vivo*. The inhibition of the $\Delta^{8,14}$-sterol reductase has been confirmed by enzymatic studies in cell-free extracts (A. Rahier, unpublished results). It has been suggested that tertiary amines can be metabolized in plants into their *N*-oxides [16]. Therefore, the detection of $\Delta^{8,14}$-sterols in plant cells treated with **1** or substituted morpholines (**4**) could reflect the metabolism of the drug inside the treated plants and could constitute a way to reveal N-oxidation reactions.

*Identical results are obtained in both bramble cell suspension cultures and maize seedlings.* Comparison of Tables 1 and 3 shows that nearly identical results are obtained when one compares the sterol profile of both bramble cell and of maize seedlings for a given sterol biosynthesis inhibitor. The only exception is represented by fenpropimorph (**4**); a morpholine derivative which was shown to inhibit powerfully the COI and the $\Delta^8 \rightarrow \Delta^7$-sterol isomerase [5, 12, 17] and which has been used as a reference molecule in the present work. Incidentally, it should be noted that the side chains of fenpropimorph (**4**) and **5** are identical. Fenpropimorph at the concentration tested is an extremely potent inhibitor of the $\Delta^8 \rightarrow \Delta^7$-sterol isomerase in bramble cells whereas it inhibits strongly the COI in maize seedlings. By contrast, **5** which possesses a similar side chain to **4** but an 8-azadecalin bicyclic system, is a potent inhibitor of the COI in both bramble cells and maize seedlings. To explain these results, it can be suggested that morpholine derivatives such as **4** or tridemorph have a higher degree of freedom and can accommodate the active sites of the COI, $\Delta^8 \rightarrow \Delta^7$-sterol isomerase and even $\Delta^{8,14}$-sterol reductase [18–20]. By contrast, the 8-azadecalin skeleton is less flexible and in addition it would be anchored to the active site of the COI by its 3$\beta$-OH group, perhaps explaining its increased selectivity towards the COI [21].

*The 4α-methyl sterol accumulation allows discrimination between the various sterol biosynthesis inhibitors.* When one considers the 4-desmethylsterol fraction from bramble cells treated with the sterol biosynthesis inhibitors listed in Table 2A, the differences in the sterol profile become more apparent. Compounds **10** and especially **2** and **5** lead to a strong accumulation of cyclopropyl sterols whereas in bramble cells treated with **1**, **4** and **9**, cyclopropyl sterols are present in low amounts and $\Delta^8$-sterols ($\Delta^{8,14}$-sterols in the case of **9**) accumulate tremendously. The situation is less clear when one considers the total sterol profile (Table 2B) since 4α-methylcyclopropyl sterols and especially cycloeucalenol (**13**) accumulate strongly. Such a situation would result from a different behaviour of the various possible 4α-methyl sterols towards the 4α-methylsterol 4α-methyl demethylase. The strong accumulation of cycloeucalenol suggests that the 4α-methylsterol 4α-methyl demethylase reaction is very slow with cycloeucalenol as a substrate. By contrast, 4α-methyl-$\Delta^8$-sterols are barely detectable although a strong accumulation of 4α-methyl-5α-ergosta-8,24(28)-dien-3$\beta$-ol (**31**) should be expected since the enzymatic step which is blocked by **1** or **4** is the conversion of **32** into its $\Delta^7$-positional isomer, namely 24-methylenelophenol (**41**) [1]. The lack of accumulation of 4α-methyl-$\Delta^8$-sterols would be consistent with the fact that these sterols are expected to be excellent substrates of the 4α-methylsterol 4α-methyl demethylase enzyme in our materials, as already shown in rat liver [23]. An intermediate situation seems to exist in the case of $\Delta^{8,14}$-sterols which accumulate undoubtedly in the 4α-methylsterol fraction from bramble cells treated

with 9 and which, therefore, could be worse substrates of the 4α-methyl demethylase than 4α-methyl $\Delta^8$-sterols. Taking into account the above considerations, one can explain the absence of cyclopropyl sterols in the 4-desmethylsterol fraction in spite of their accumulation in the 4α-methylsterol fraction (bramble cells treated with **1** and **4**) by a competition between 4α-methylcyclopropyl- and 4α-methyl-$\Delta^8$-($\Delta^{8,14}$)-sterols towards the 4α-methylsterol demethylase. This competition, being in favour of $\Delta^8$-sterols, would strongly hinder the demethylation of cyclopropyl sterols. In the cases of **2**, **5** and **10**, the COI being completely blocked, there would be no more $\Delta^8$-sterol available to compete with cyclopropyl sterols and the demethylation of these latter sterols should become apparent.

Finally, 8-azadecalins and their *N*-oxide derivatives are useful molecular tools to manipulate the sterol content of plant membranes. Depending on the alkyl substituent carried by the nitrogen atom and the inhibitor concentration, the relative proportions of 9β,19-cyclopropyl-, $\Delta^5$- and $\Delta^8$-sterols can be modulated. The *N*-oxide derivatives of these tertiary amines orientate the biosynthetic flow towards accumulation of $\Delta^{8,14}$-sterols. Therefore, by acting on these three parameters, it becomes possible to obtain plant cells with a predetermined sterol profile. This is an important opportunity to study the effects of cyclopropyl-, $\Delta^8$- or $\Delta^{8,14}$-sterols in living cells. A study of the mechanism of the inhibition using cell-free extracts and more purified enzymatic systems is in progress.

## EXPERIMENTAL

Most of the techniques and the materials used in this work have been thoroughly described previously [1, 6, 7, 24, 25].

*Plant material.* Bramble cells suspension cultures were grown under continuous white light at 25° on a synthetic sterile medium as described previously [26]. The various sterol biosynthesis inhibitors (0.5–20 mg/l.) were added in EtOH soln to the culture medium. The drug soln was sterilized by ultrafiltration. Control maize (var. LG 11) seedlings were allowed to germinate in moist vermiculite in light at 25° for 2 weeks. The sterol biosynthesis inhibitors were dissolved in $H_2O$ and the caryopses were soaked for 8 hr in the soln. The caryopses were germinated and grown in vermiculite in the same way as the controls except that the vermiculite was continuously soaked with the inhibitor soln (1 l./day, 100 seedlings) in place of pure $H_2O$. Plants were measured after various times of growth, and the mean height and standard deviation of the mean for each treatment were calculated.

*Sterol biosynthesis inhibitors.* *N*-Benzyl-8-aza-4α,10-dimethyl-*trans*-decal-3β-ol (**1**) and *N*-dodecyl-8-aza-4α,10-dimethyl-*trans*-decal-3β-ol (**10**) were synthesized as described previously [1]. The syntheses of *N*([1,5,9-trimethyldecyl]-4α,10-dimethyl-8-aza-*trans*-decal-3β-ol (**2**), *N*-[3-]4-tert-butylphenyl-[2-methyl]-propyl-8-aza-4α,10-dimethyl-*trans*-decal-3β-ol (**5**) and *N*-oxide-4α,10-dimethyl-*trans*-decal-3β-ol (**9**) will be described elsewhere [22]. Fenpropimorph: 4-(3-)4-tert-butylphenyl-(2-methyl)-propyl-2,6-dimethyl morpholine (**4**) and its *N*-oxide derivative (**8**) were gifts from Dr. Pommer (B.A.S.F., West Germany).

*Authentic materials.* Cycloeucalenol was extracted from tallow wood (*Eucalyptus mycrocorys*) kindly provided by Prof. R. A. Massy Westropp (Adelaide, Australia). Cycloartenol (**11**) was kindly supplied by Dr. A. S. Narula (Durham, U.S.A.) and Prof. G. Ourisson (Strasbourg, France). Cyclolaudenol was a generous gift from Prof. D. Arigoni (Zürich, Switzerland). 24-Methylpollinastanol (**16**) was extracted from maize roots treated with fenpropimorph as described previously [7]. 24-Methylenepollinastanol (**15**) was extracted from bramble cell suspension cultures treated with fenpropimorph [6]. (24*R*)-24-Ethyl-5α-cholest-8-en-3β-ol (**26**) and 5α-stigmasta-8,*Z*-24(28)-dien-3β-ol (**25**) were isolated from bramble cell suspension cultures treated with AY 9944 [24].

*Analytical procedure.* Bramble cells and maize seedlings grown in the presence of the inhibitors were treated as described previously [1, 7, 23, 24]. This procedure led to the isolation of 4,4-dimethyl-, 4α-methyl- and 4-desmethylsteryl acetates. Each of the three classes of sterol acetates was analysed by GC with a GC equipped with a FID and a silica fused capillary column (WCOT, 25 m × 0.25 mm i.d.) coated with OV[1] ($H_2$ flow: 2 ml/min). The temp. programme used included a fast rise from 60° to 230° (30°/min), then a slow rise from 230° to 280° (2°/min). The total amount of sterols present in each class was quantified using an integrator. Analytical argentation TLC (15% $AgNO_3$ impregnated silica gel; cyclohexane–toluene (7:3); migration for 15 hr) was performed on each class of steryl acetate and the bands obtained were analysed by GC. There were three bands of 4,4-dimethylsteryl acetates in the case of both control cells and treated cells corresponding, in order of decreasing polarity, to **12**-acetate, an unidentified tetracyclic triterpene, **11**-acetate and a mixture of α- and β-amyrin acetates. There were seven bands of 4α-methylsteryl acetates from bramble cells or maize seedlings treated with inhibitors. The first band at very low $R_f$ (0.1) corresponded to products identified as **30**-and **31**-acetates. The second band ($R_f$ 0.3) contained **32**-acetate. The third band ($R_f$ 0.35) contained generally enormous amounts of **13**-acetate. The fourth band ($R_f$ 0.40), the fifth band ($R_f$ 0.55), the sixth band ($R_f$ 0.60) and the seventh band ($R_f$ 0.75) contained **33**-, **14**-, **27**- and **34**-acetates, respectively. **34**-acetate was particularly abundant in maize seedlings. There were seven bands in the 4-desmethylsteryl acetates. The first band at $R_f$ (0.10) contained **19**- and **21**-acetates. The second band ($R_f$ 0.25) corresponded to a mixture of **20**-, **22**-**23**- and **27**-acetates. The third band ($R_f$ 0.40) contained **15**-, **25**-, and **29**-acetates. The fourth band ($R_f$ 0.45) corresponded to **17**-acetate. The fifth band ($R_f$ 0.60) contained the mixture of $\Delta^5$-steryl acetates: **28**-, **29**- and **37**-acetates; the sixth band ($R_f$ 0.65) corresponded to a mixture of $\Delta^8$-steryl acetates: **24**-, **26**- and **36**-acetates; and the seventh band ($R_f$ 0.70) contained a mixture of 9β,19-cyclopropyl sterols: **16**- and **18**-acetates. As bands 5, 6 and 7 were poorly resolved, some further separation was necessary. This was possible after saponification of the mixture of acetates of **28**, **29**, **37**, **24**, **27**, **36**, **16** ($AgNO_3$ impregnated alumina, unwashed $CHCl_3$ as developing solvent, migration 1 hr). This technique separated $\Delta^5$-sterols from the mixture of $\Delta^8$- and cyclopropyl sterols. As the GC separated without ambiguity all components of the mixture of $\Delta^8$- and cyclopropyl steryl acetates, no further purification test was generally performed. When the isolation of pure cyclopropyl sterols was necessary, the mixture of $\Delta^8$- and cyclopropyl steryl acetates was treated with a saturated soln of *p*-nitroperbenzoic acid in $Et_2O$ (1 ml) overnight at room temp. The excess of peracid was destroyed by adding KI, then $NaS_2O_3$ (5% in $H_2O$) was added and the mixture was extracted with hexane [10]. After conventional work-up the mixtures of steryl acetates was submitted to TLC (silica gel) in which $CH_2Cl_2$ was the developing solvent and the cyclopropyl steryl (**16** and **18**) acetates were unambiguously separated from the epoxides of $\Delta^8$ (**24**, **26** and **36**) acetates. All the sterols isolated from inhibitor-treated bramble cells and maize seedlings were identified by their $^1H$ NMR and mass spectra as described previously [1, 7, 10, 23, 24]. These data, being strictly identical to those already published, have not been detailed here.

*Nomenclature.* Cycloartenol = 4,4,14α-trimethyl-9β,19-cyclo-5α-cholest-24-en-3β-ol (**11**); 24-methylenecycloartanol = 4,4,14α-

trimethyl-9β,19-cyclo-5α-ergost-24(28)-en-3β-ol (**12**); cycloeucalenol = 4α,14α-dimethyl-9β,19-cyclo-5α-ergost-24(28)-en-3β-ol (**13**); obtusifoliol = 4α,14α-dimethyl-5α-ergosta-8,24(28)-dien-3β-ol (**40**); cyclofontumienol = 4α,14α-dimethyl-9β,19-cyclo-5α-stigmast-*Z*-24(28)-en-3β-ol (**14**); 31-norcyclobranol = 4α,14α-dimethyl-9β,19-cyclo-5α-ergosta-8,24-dien-3β-ol (**27**); 24-methylenelophenol = 4α-methyl-5α-ergosta-7,24(28)-dien-3β-ol (**41**); 24-methylenepollinastanol = 14α-methyl-9β,19-cyclo-5α-ergost-24(28)-en-3β-ol (**15**); 24-methylpollinastanol = 14α-methyl-9β,19-cyclo-5α-ergostan-3β-ol (**16**); 24-ethylidenepollinastanol = 14α-methyl-9β,19-cyclo-5α-stigmast-*Z*-24(28)-en-3β-ol (**17**); 24-ethylpollinastanol = 14α-methyl-9β,19-cyclo-5α-stigmastan-3β-ol (**18**); fecosterol = 5α-ergosta-8,24(28)-dien-3β-ol (**23**); 24-methylenecholesterol = ergosta-5,24(28)-dien-3β-ol (**27**); campesterol = (24*R*)-24-methylcholest-5-en-3β-ol (**28**); isofucosterol = stigmasta-5,*Z*-2(28)-dien-3β-ol (**38**); sitosterol = (24*R*)-24-ethylcholest-5-en-3β-ol (**29**); stigmasterol = (24*R*)-24-ethylcholesta-5,*E*-22-dien-3β-ol (**37**).

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