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# INHIBITION OF $\Delta^8 \rightarrow \Delta^7$-STEROL ISOMERASE AND OF CYCLOEUCALENOL–OBTUSIFOLIOL ISOMERASE BY *N*-BENZYL-8-AZA-4α,10-DIMETHYL-*TRANS*-DECAL-3β-OL, AN ANALOGUE OF A CARBOCATIONIC HIGH ENERGY INTERMEDIATE

ALAIN RAHIER, MARYSE TATON, PAULETTE SCHMITT, PIERRE BENVENISTE, PIERRE PLACE* and CLAUDE ANDING*

Laboratoire de Biochimie Végétale, Institut de Botanique, 67083 Strasbourg, France; *Rhône-Poulenc Agrochimie, Centre de Recherche de la Dargoire, 69263 Lyon Cedex 09, France

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**Key Word Index**—*Rubus fruticosus*; Rosaceae; *Zea mays*; Gramineae; *N*-benzyl-8-aza-4α,10-dimethyl-*trans*-decal-3β-ol; high energy intermediate analogues; inhibition of $\Delta^8 \rightarrow \Delta^7$-sterol isomerase and of cycloeucalenol–obtusifoliol isomerase.

**Abstract**—An enzymatic assay for the $\Delta^8 \rightarrow \Delta^7$-sterol isomerase, an enzyme involved in sterol biosynthesis, has been developed in higher plants. This assay has been used in the study of various inhibitors. *N*-Benzyl-8-aza-4α,10-dimethyl-*trans*-decal-3β-ol was designed to mimic the C-8 and the C-9 carbocationic high energy intermediates occurring during the reactions catalysed by the $\Delta^8 \rightarrow \Delta^7$-sterol isomerase and the cycloeucalenol obtusifoliol isomerase, respectively. In accordance with the 'transition state analogues' theory, this analogue of a high energy intermediate was found to be a very potent and specific inhibitor of the two enzymatic reactions both *in vitro* and *in vivo*.

## INTRODUCTION†

One major feature of several enzymes of sterol biosynthesis is that they catalyse reactions involving postulated or demonstrated carbocationic high energy intermediates (HEI). This is so with farnesyl pyrophosphate–squalene synthetase [1], 2(3)-epoxy-2(3)-dihydrosqualene cyclase [2], sterol-C-24- and C-28-methyltransferases [3], cycloeucalenol–obtusifoliol isomerase (COI) [4] and $\Delta^8 \rightarrow \Delta^7$-sterol isomerase [5]. In order to investigate the potent inhibitors of these enzymes we have researched the design and synthesis of analogues of the HEIs occurring in the catalytic pathway of these enzymatic reactions. Theoretical considerations have established that catalysis of a reaction by an enzyme implies that the activated form of the substrate, occurring during the reaction pathway, is bound more energetically by the active site than the substrate in its ground state [6, 7]. It results that molecules which bear structural and electronic resemblances to such metastable intermediates should be very strong inhibitors of the corresponding catalysed reactions [7–9]. Such a strategy has been experimented with success in our group with the synthesis of (24-*R*,*S*)-24-methyl-25-azacycloartanol. This compound, an analogue of an HEI involved in the reaction catalysed by the *S*-adenosyl-(L)-methionine cycloartenol-C-24-methyltransferase (AdoMet–CMT), was shown to be a very potent inhibitor of this enzyme [10]. Similar concepts have been used to design inhibitors of farnesyl pyrophosphate–squalene synthetase [11] and of 2(3)-epoxy-2(3)-dihydrosqualene-β-amyrin cyclase [12].

We have focused our attention on the COI and the $\Delta^8 \rightarrow \Delta^7$-sterol isomerase (Fig. 1). COI catalyses the cleavage of the 9β,19-cyclopropane ring of cycloeucalenol (**1**) to give obtusifoliol (**2**). The first step of this reaction consists of the C-19 protonation of cyclopropane leading to the HEI (**3a**) bearing a carbocation at C-9. Then there is a *cis* regio-specific elimination of the H-8β to give the $\Delta^8$-double bond [4]. To explain these stereochemical features, it has been suggested that the HEI (**3a**) could be stabilized by a suitable sub-site (anionic or electron-rich sub-site) of the enzyme [4, 13].

The $\Delta^8 \rightarrow \Delta^7$-sterol isomerase catalyses the $8 \rightarrow 7$ isomerization of the $\Delta^8$-double bond of a $\Delta^8$-sterol such as **4** to give the $\Delta^7$-sterol (**5**) (Fig. 1). According to Wilton *et al.* [14], the reaction involves first an α-protonation of the $\Delta^8$-double bond giving an HEI (**6**) possessing a carbocation at C-8 (a localized form of a three centres $H^+$ bridging a C-8–C-9 bond). Then there is an elimination of a C-7 proton to give the $\Delta^7$-double bond. Loss of the 7β-hydrogen atom occurs in rat liver homogenate [15], whereas in yeast the enzyme action proceeds with loss of the 7α-hydrogen atom [16, 17]. In higher plants, *in vivo* experiments suggest that the H-7β is eliminated, as in rat liver [3, 18]. To our knowledge no experiments using cell-free extracts have been reported to study the isomerase in higher plants. With the above considerations in mind we have designed model molecules, such as *N*-benzyl-8-aza-

†Terminology: cycloartenol (**23**), 4,4,14α-trimethyl-9β,19-cyclo-5α-cholest-24-en-3β-ol; cycloeucalenol (**1**), 4α,14α-dimethyl-9β,19-cyclo-5α-ergost-24(28)-en-3β-ol; 24-methylene pollinastanol (**11**), 14α-methyl-9β,19-cyclo-5α-ergost-24(28)-en-3β-ol; cyclofontumienol (**12**), 4α,14α-dimethyl-9β,19-cyclo-5α-stigmast-*Z*-24(28)-en-3β-ol; obtusifoliol (**2**), 4α,14α-dimethyl-5α-ergosta,8,24(28)-dien-3β-ol; 24-methylenelophenol, 4α-methyl-5α-ergosta-7,24(28)-dien-3β-ol; isofucosterol (**17**), stigmasta-5, *Z*-24(28)-dien-3β-ol; sitosterol (**19**), (24-*R*)-24-ethyl-cholest-5-en-3β-ol; campesterol (**18**), (24-*R*)-24-methyl-cholest-5-en-3β-ol.

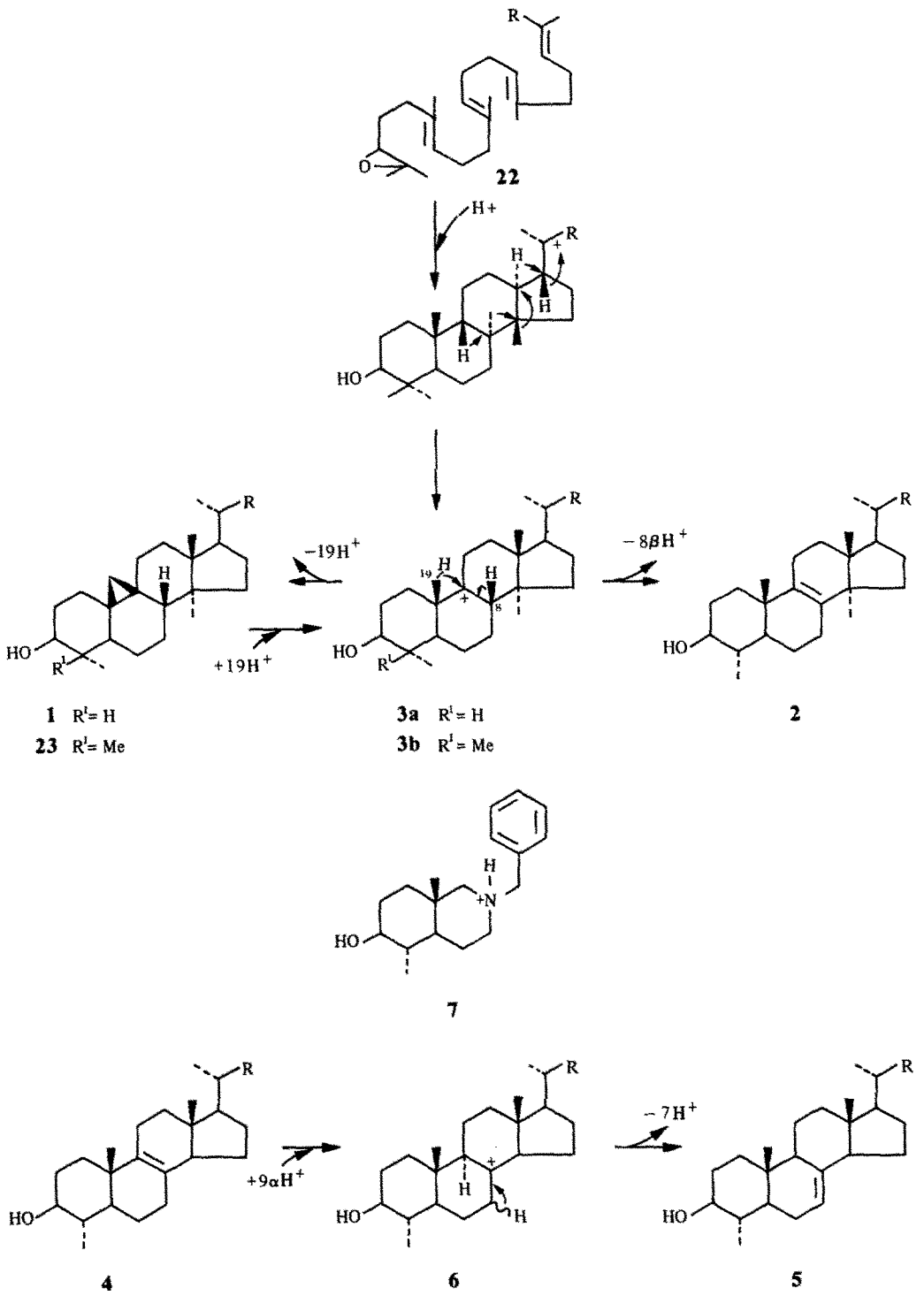



Fig. 1. Hypothetical reaction pathways for cycloeucalenol–obtusifoliol isomerase (**1** → **3a** → **2**), $\Delta^8 \rightarrow \Delta^7$-sterol isomerase (**4** → **6** → **5**) and 2(3)-epoxy-2(3)-dihydrosqualene–cycloartenol cyclase (**22** → **3b** → **23**).

4α,10-dimethyl-*trans*-decal-3β-ol (**7**), which can be considered as low $M_r$ analogues of HEIs occurring in the reactions catalysed by the COI and the isomerase. Indeed, these tertiary amines ($pK_a$ 9–10), being essentially protonated under physiological conditions, present evident structural and electronic similarities with the HEI(s) (**3a** and **6**) involved during the reactions catalysed by the two enzymes (Fig. 1). In particular, the location of the positive charge conferred by the ammonium group in **7** is identical to that of the C-8 carbocation of the HEI (**6**). On the other

hand, the location of the positive charge in **7** is spatially very close to that occupied by the carbocation at C-9 in the HEI (**3a**). Accordingly, we hoped that **7** and its derivatives could be inhibitors of the COI and of the $\Delta^8 \rightarrow \Delta^7$-sterol isomerase. This study deals with the effect of **7** on the sterol profile of suspensions of bramble (*Rubus fructicosus* L.) cells and with the inhibition of both the COI and the $\Delta^8 \rightarrow \Delta^7$-sterol isomerase in cell-free extracts from maize (*Zea mays* L.) seedlings. The results show that **7** is an extremely potent inhibitor of these enzymes both *in vivo* and *in vitro*. A preliminary communication of this work has been published previously [19].

## RESULTS

### *Effect of* 7 *on the sterol profile of suspensions of bramble cells*

Suspensions of bramble cells ($2 \times 10^4$ cells/ml) were grown in the presence of **7** (1–5 mg/l.). No effect on growth was observed in this concentration range but a slight growth inhibition was observed at higher concentrations (10 mg/l.). When the stationary phase was reached, the cells were harvested and the sterols were analysed. In agreement with previous studies [20–22], the major sterols of control cells were $\Delta^5$-sterol, campesterol and isofucosterol constituting 96% of total sterols (Table 1). In cells treated with **7**, the concentration of $\Delta^5$-sterols decreased strongly, whereas new sterols accumulated (Table 1). When the cells where treated with the lowest concentration (1 mg/l.) of **7**, $\Delta^8$-sterols predominated strongly (87% of total sterols). Among them, (24-*R*)-24-ethyl-5α-cholest-8-en-3β-ol (**8**) and 5α-stigmasta-8, *Z*-24(28)-dien-3β-ol (**9**) were the most abundant sterols. In addition, 4α-methyl-5α-ergosta-8,24(28)-3β-ol (**10**) and 4α-methyl-5α-stigmasta-8,*Z*-24(28)-dien-3β-ol (**4**) were also identified. When the concentration of **7** increased, the content of $\Delta^8$-sterols decreased whereas 9β,19-cyclopropyl sterols, such as cycloeucalenol (**1**), 24-methylene pollinastanol (**11**) and cyclofuntumienol (**12**) accumulated. At higher concentrations of **7** (10 mg/l.), 9β,19-cyclopropyl sterols became more than 50% of the total sterols (data not shown). Finally, low amounts of $\Delta^{8,14}$-sterols, such as (24- ξ)-24-methyl-5α-cholesta-8,14-dien-3β-ol (**13**) and (24-ξ)-24-ethyl-5α-cholesta-8,14-dien-3β-ol (**14**) were detected at the highest concentrations of **7** used. The new sterols have been identified by NMR and mass spectrometry as described previously [20–23]. These results did indicate that **7** interfered with sterol biosynthesis and suggested that the major targets of the drug were the $\Delta^8 \rightarrow \Delta^7$-sterol isomerase and the COI.

### *Inhibition by* 7 *of the COI and of the* $\Delta^8 \rightarrow \Delta^7$*-sterol isomerase in cell-free extracts from maize*

To give further support to the above results, the inhibition of COI and $\Delta^8 \rightarrow \Delta^7$-sterol isomerase activities by **7** was measured *in vitro* in an enzymatic system

Table 1. Sterols of control and *N*-benzyl-8-aza-4α,10-dimethyl-*trans*-decal-3β-ol (**7**)-treated bramble cells*

| | | 7 (mg/l.) | | |
|---|---|---|---|---|
| | | Control | Treated | |
| | $RR_t$/cholesterol | 0 | 1 | 5 |
| Cycloartenol (**23**) | 1.46 | trace | trace | 0.5 |
| 24-Methylene cycloartanol | 1.55 | 0.5 | 0.5 | 3.0 |
| Cycloeucalenol (**1**) | 1.46 | trace | 3 | 20 |
| Obtusifoliol (**2**) | 1.38 | trace | trace | 0.2 |
| 4α-Methyl-stigmasta-8,*Z*-24(28)-dien-3β-ol (**4**) | 1.54 | 0 | 0.3 | 0.5 |
| 4α-Methyl-stigmasta-7,*Z*-24(28)-dien-3β-ol (**5**) | 1.61 | trace | 0 | 0 |
| Cyclofuntumienol (**12**) | 1.62 | 0 | 0 | 0.5 |
| 24-Methylene pollinastanol (**11**) and 24-methyl pollinastanol (**29**) | 1.38 | 0 | 1.0 | 5.5 |
| (24ξ)-24-Methyl-5α-cholesta-8,14-dien-3β-ol (**13**) | 1.33 | 0 | 0 | 1 |
| (24*R*)-24-Ethyl-5α-cholesta-8,14-dien-3β-ol (**14**) | 1.50 | 0 | 0 | 3 |
| 5α-Ergosta-8,24(28)-dien-3β-ol (**15**) | 1.32 | 0 | 0.1 | 0.2 |
| 5α-Stigmasta-8,*Z*-24(28)-dien-3β-ol (**9**) | 1.47 | 0 | 35 | 15 |
| (24ξ)-24-Methyl-5α-cholest-8-en-3β-ol (**16**) | 1.33 | 0 | 4 | 4 |
| (24*R*)-24-Ethyl-5α-cholest-8-en-3β-ol (**8**) | 1.50 | 0 | 48 | 40 |
| Isofucosterol (**17**) | 1.44 | 12 | 2 | 0.4 |
| Campesterol (**18**) | 1.29 | 14 | 0.3 | 0 |
| Sitosterol (**19**) | 1.43 | 70 | 5.5 | 1 |
| Unknown sterols, α- and β-amyrins | | 3 | 1 | 4 |
| $\Delta^5$-Sterols | — | 96 | 8 | 1.5 |
| $\Delta^8$-Sterols | — | 0 | 87 | 60 |
| $\Delta^{8,14}$-Sterols | — | 0 | 0 | 4 |
| 9β,19-Cyclopropyl sterols | — | 1 | 4.5 | 30 |

*As a percentage of total sterols.

prepared from maize seedlings as described earlier [4, 24]. In the case of the COI, the microsomes were incubated in the presence of cycloeucalenol (**1**) at 30° for 45 min. The product of the reaction, obtusifoliol (**2**), was identified and quantified as described previously [4] using GC/MS as detailed in the Experimental. A preliminary study of the $\Delta^8 \rightarrow \Delta^7$-sterol isomerase [M. Taton, unpublished results] as well as biosynthetic considerations [20] have given evidence that 4α-methyl-5α-stigmasta-8,Z-24(28)-dien-3β-ol (**4**) was a good substrate of the enzyme. Therefore, the microsomes were incubated in the presence of **4** at 30° for various periods of time. The product of the reaction, 4α-methyl-5α-stigmasta-7,Z-24(28)-dien-3β-ol (**5**) was identified by GC/MS and $^1$H NMR as detailed in the Experimental. Thus, for the first time to our knowledge a $\Delta^8 \rightarrow \Delta^7$-sterol isomerase activity was measured in a cell-free extract from higher plant cells. Compound **7** was shown to inhibit strongly both the COI and the $\Delta^8 \rightarrow \Delta^7$-sterol isomerase activities. From the inhibition curves it was possible to calculate $I_{50}$ (inhibitor concentration required to reduce the reaction velocity by half) values of 0.1 and 0.13 μM for the COI and the $\Delta^8 \rightarrow \Delta^7$-sterol isomerase, respectively (Table 2). Under the assay conditions used in this study, where the concentrations of the substrates were close their $K_m$ values (100 μM for both **1** and **4**), $I_{50}$ values are in the order of the inhibition constants [25] and the values obtained for **7** indicate the COI and the $\Delta^8 \rightarrow \Delta^7$-sterol isomerase had a much higher affinity (2–3 orders of magnitude) for the HEI analogue than for their best substrate **1** and **4**, respectively. To gain more information about the molecular features involved in the inhibition, **7** has been compared to 8-aza-4α,10-dimethyl-*trans*-decal-3β-ol (**20**) and to 4α,10-dimethyl-*trans*-decal-3β-ol (**21**). Compound **20** differs from **7** in lacking a benzyl substituent on the nitrogen atom. Compound **21** is a neutral isosteric analogue of **20**. The following conclusions can be drawn from the data of Table 2: (i) whereas **20** inhibited the COI and the $\Delta^8 \rightarrow \Delta^7$-sterol isomerase, **21** was not active at all on both the enzymes; and (ii) the presence of a benzyl substituent on the nitrogen atom increased the inhibitory power by a factor of 50.

*Specificity of the inhibition by* **7**

Table 2 gives the results of a comparative study of the inhibition by **7**, **20** and **21** of COI, $\Delta^8 \rightarrow \Delta^7$-sterol isomerase, 2(3)-epoxy-2(3)-dihydrosqualene (**22**)–cycloartenol (**23**) cyclase (ESCC) [2] and the AdoMet–CMT [10]. The ESCC was chosen since its reaction mechanism in its latter stage also involved a C-9 carbocationic HEI (**3b**) [3] similar to those involved in the reaction mechanism of the two former enzymes (Fig. 1). The latter enzyme was considered since its reaction mechanism involves a C-25 carbocationic HEI [10] very different in its structure from those involved during the reactions catalysed by the three other enzymes. The data obtained (Table 2) show that: (i) under conditions where the COI and the $\Delta^8 \rightarrow \Delta^7$-sterol isomerase were strongly inhibited by **7** and **20**, neither the ESCC nor the AdoMet–CMT were inhibited by these two drugs; (ii) **7** and **20** inhibited the $\Delta^8 \rightarrow \Delta^7$-sterol isomerase as strongly as the COI; and (iii) decalin (**21**) did not inhibit any of the enzyme tested.

*Structure–activity relationships relative to the inhibition of COI by* **7** *and its derivatives*

The structural features which have been considered are: (i) the nature of the alkyl substituent carried by the nitrogen atom (compounds **7**, **20** and **24**–**27**); and (ii) the number of the methyl groups present at C-4 (compounds **7** and **28**). The results obtained (Table 3) showed that: (i) the substitution of the aromatic ring has only little effect on the inhibitory activity, however, the *m*-trifluoromethyl derivative (**26**) was significantly less potent than the *p*-methyl- and *p*-chloro derivatives (**24** and **25**); (ii) replacement of the benzyl substituent of **7** by a dodecyl group (**27**) has no effect on the inhibitory

Table 2. Inhibition of enzymes of sterol biosynthesis by 8-aza-decalins (**7** and **20**) and by 4α,10-dimethyl-*trans*-decal-3β-ol (**21**)

| Inhibitors | Cycloeucalenol–obtusifoliol isomerase (COI) $I^*_{50}$ (μM) | $\Delta^8 \rightarrow \Delta^7$-Sterol isomerase $I^*_{50}$ (μM) | 2,3-Epoxy-2,3-dihydrosqualene–cycloartenol cyclase (ESCC) $I^*_{50}$ (μM) | AdoMet–cycloartenol–C-24-methyltransferase (AdoMet–CMT) $I^*_{50}$ (μM) |
|---|---|---|---|---|
| **7** | 0.10±0.05† | 0.13±0.05‡ | >200§ | 50 |
| **20** | 17.5 | 10.0 | >200 | 200 |
| **21** | —‖ | —‖ | —¶ | —‖ |

In all the enzymatic assays, the microsomes (0.5 ml) were incubated in the presence of substrate (100 μM), various concentrations of inhibitors (10 nM–100 μM) and Tween-80 [final concentration 0.1% (w/v)] at 30° for 45 min.

*$I_{50}$, Inhibitor concentration required to reduce the reaction velocity by half.

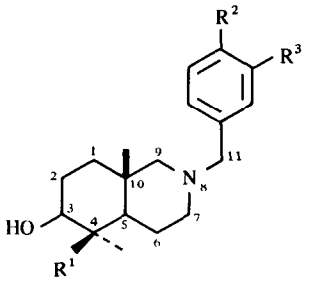
†Average of six determinations.

‡Average of three determinations.

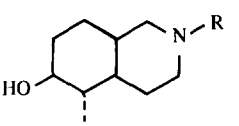
§An identical result (no inhibition) was obtained when **28** was used as inhibitor.

‖No inhibition at the highest concentration (100 μM) tested.

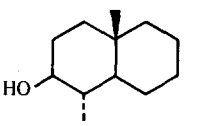
¶Inhibition 25% at the highest concentration (100 μM) tested. The use of **21** as inhibitor of the ESCC in rat liver homogenate gave an $I_{50}$ value of 1 μM [31] in agreement with ref. [32].



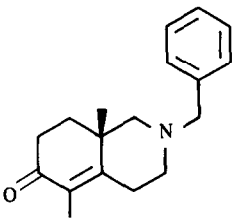
**7** $R^1 = H, R^2 = R^3 = H$
**24** $R^1 = H, R^2 = Me, R^3 = H$
**25** $R^1 = H, R^2 = Cl, R^3 = H$
**26** $R^1 = H, R^2 = H, R^3 = CF_3$
**28** $R^1 = Me, R^2 = R^3 = H$



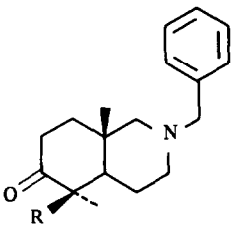
**20** R = H
**27** $R = nC_{12}H_{25}$



**21**



**30**



**31** R = H
**32** R = Me

Fig. 2. Chemical structures of *N*-benzyl-8-aza-4α,10-dimethyl-*trans*-decal-3β-ol (**7**) and its derivatives.

Table 3. Inhibition of COI by various 8-aza decalins possessing different alkyl substituents on the nitrogen atom

| | Inhibitors [$I_{50}^*$ (μM)] | | | | | |
|---|---|---|---|---|---|---|
| | **7** | **24** | **25** | **26** | **27** | **28** |
| Experiment 1 | 0.035 | 0.030 | 0.030 | 0.050 | 0.035 | — |
| Experiment 2 | 0.080 | — | — | — | — | 0.800 |

The enzyme assays were performed as described in Table 2.
*$I_{50}$, Inhibitor concentration required to reduce the reaction velocity by half.

capacity; and (iii) the 4,4-dimethyl derivative (**28**) was 10 times less active than the 4α-methyl homologue (**7**).

## DISCUSSION

Our results show that in bramble suspension cultures growing in the presence of **7** (5 mg/l.), only a small amount (1.5%) of the sterols present were $\Delta^5$-sterols and most (87%) were $\Delta^8$-sterols (such as **8** and **9**) and 9β,19-cyclopropyl sterols (such as **1** and **11**). These results suggest that the major targets of **7** in the bramble cells were COI and $\Delta^8 \rightarrow \Delta^7$-sterol isomerase. As low amounts of $\Delta^{8,14}$-sterols (**13** and **14**) were also detectable, it could be suggested that a secondary target of **7** could be the $\Delta^{8,14}$-sterol $\Delta^{14}$-reductase. In order to show if these effects were due to **7** or to a metabolite, we tested the activity of this molecule *in vitro* with a cell-free system from maize seedlings. The results show unequivocally that **7** inhibits strongly the COI and the $\Delta^8 \rightarrow \Delta^7$-sterol isomerase whereas two other enzymes involved in higher plant sterol biosynthesis (the ESCC and AdoMet–CMT) are not inhibited. Therefore, it can be concluded that the COI and the $\Delta^8 \rightarrow \Delta^7$-sterol isomerase are the major targets of the sterol biosynthesis pathway of **7** *in vivo* and *in vitro*. Other cellular targets not related to sterol biosynthesis cannot be excluded at present, however, if they exist they do not affect cell growth under our experimental conditions. Possible biosynthetic pathways leading to sterols in bramble cells treated with **7** are represented in Fig. 3.

The inhibition of both COI and $\Delta^8 \rightarrow \Delta^7$-sterol isomerase by **7** results probably from the fact that, at physiological pH, **7** mimics carbocationic HEIs (**3** and **6**, respectively) involved during reactions catalysed by these two enzymes (Fig. 1). Indeed, **7** has a bicyclic structure presenting close electronic and stereochemical similarities with the A,B cycles of **3** and **6**. In particular, **7** possesses a positive charge conferred by a tertiary ammonium group which is located at a position corresponding to C-8 of **6**. The fact that the amine (**20**) inhibits both the COI and the $\Delta^8 \rightarrow \Delta^7$-sterol isomerase, whereas its neutral isosteric analogue (**21**) is completely devoid of activity, emphasizes the importance of the positive charge on the inhibitory capacity. As **7** is closest structurally to the HEI (**6**) it would be expected that **7** should inhibit more strongly $\Delta^8 \rightarrow \Delta^7$-sterol isomerase than COI. Data obtained from *in vivo* studies indeed support this view (Table 1); however *in vitro* experiments which showed no significant differences between the $I_{50}$ corresponding to the inhibition of the two enzymes by **7** (Table 2), did not confirm the *in vivo* results. This could result from the fact that ammonium groups are soft or delocalized cations. According to recent calcu-

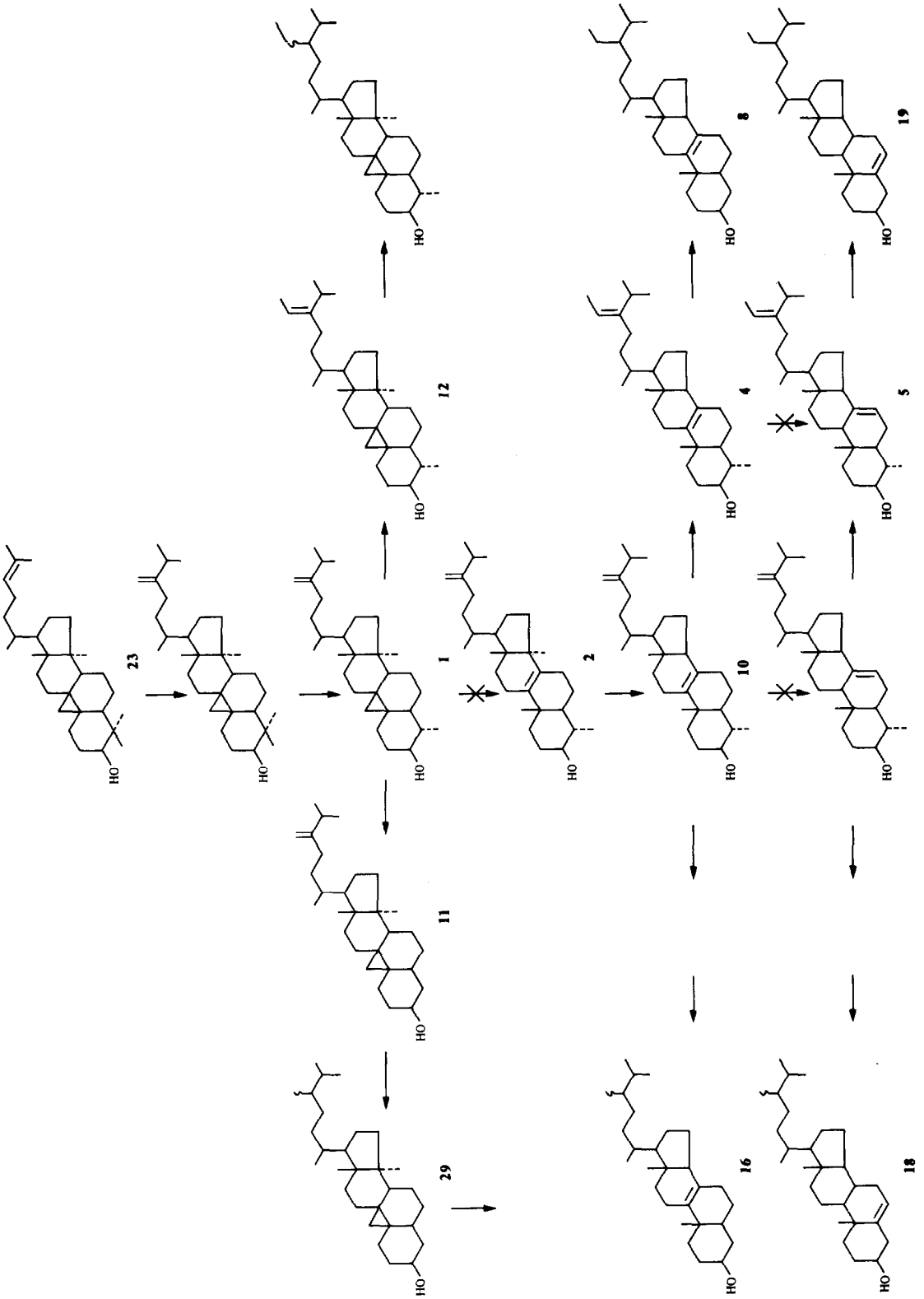



Fig. 3. Hypothetical biosynthetic scheme leading to sterols in suspensions of bramble cells treated with 7.

lations [26, 27], the ammonium charge would be distributed amongst the C-9, N-8, C-7 and benzylic hydrogens of **7**; this could explain why **7** is able to mimic the structurally close HEIs **3a** and **6** and, possibly, HEIs resulting from the protonation of the $\Delta^{14}$-double bond of $\Delta^{8,14}$-sterols. The fact that **7** and **28** do not inhibit the ESCC at all whereas they inhibit strongly COI and $\Delta^8 \rightarrow \Delta^7$-sterol isomerase is rather surprising since the three enzymic reactions are supposed to involve structurally similar C-8, C-9 carbocationic HEIs such as **3a** or **3b** which can be mimicked also by **7** and **28**. To explain these results, one can propose the following comments: in the case of the $\Delta^8 \rightarrow \Delta^7$-sterol isomerase, the enzyme conformation which stabilizes the C-8 carbocationic HEI (**6**) (Fig. 1) is probably not very different from that which binds the substrate (**4**) in its ground state since the geometries of **4** and **6** are similar. By contrast, the substrate of the ESCC (2,3-epoxy-2,3-dihydrosqualene, **22**, an acyclic, conformationally flexible molecule) is profoundly different from the C-8, C-9 carbocationic HEI (**3b**) (a polycyclic, conformationally rigid intermediate), therefore, the enzyme conformation which stabilizes the C-8 HEI, must be very different from that which binds **22**. In order to bind **7**, the ESCC would have to attain the appropriate conformation. As discussed by several authors [28, 29], this process could be very slow, possibly explaining why the inhibition of the ESCC by **7** and **28** was not observed in our experimental conditions. Other explanations have also been proposed, consisting particularly of speculations regarding the concerted character of the reaction catalysed by the ESCC [30, 31], the COI [19] and the $\Delta^8 \rightarrow \Delta^7$-sterol isomerase and correlatively with the stability of the carbocationic species involved in the reaction pathways leading to **23**, **2** and **5**, respectively.

Finally, some other molecular parameters of the inhibition of the COI by 8-aza-decalins have been determined. (i) The presence of a benzyl substituent on the nitrogen atom increased by a factor of 50 the inhibitory power. The benzyl substituent can be replaced by a dodecyl substituent without loss of activity. This observation stresses the importance of a hydrophobic substituent on the nitrogen atom in order to optimize the inhibition. (ii) Inducers–electron donors (*p*-Me, p-Cl) substituents on the benzyl group did not increase significantly the inhibitory power whereas electron attractor (*p*-$CF_3$) substituents decreased the inhibitory capacity of the aza-decalin. (iii) The presence of a 4$\beta$-methyl group resulted in a strong decrease of the inhibitory capacity. This is in agreement with results previously obtained [33, 34] where it was shown that 4,4-dimethyl- and 4$\beta$-methyl cyclopropyl sterols were not substates of the COI and this underlines the fact that steric constraints would also exist at the 4$\beta$-methyl level during interaction of the enzyme with HEI (**3a**).

To summarize, the present work describes the inhibition of $\Delta^8 \rightarrow \Delta^7$-sterol isomerase and COI by 8-aza-decalins, such as **7**. These latter have been used as model molecules of relatively low $M_r$, capable of mimicking carbocationic HEIs involved in the reaction mechanisms of both $\Delta^8 \rightarrow \Delta^7$-sterol isomerase and COI. They have been shown to inhibit strongly ($K_i/K_m = 10^{-3}$) these two enzymes and to interfere with sterol biosynthesis. Therefore, 8-aza-decalins, such as **7**, can be considered as leader molecules of a new class of rationally designed inhibitors. These compounds could be of value for agronomical applications as potential fungicides.

## EXPERIMENTAL

Most of the techniques and the materials used in this work have been described thoroughly previously [20–24]. Only details relevant to the present work will be given here.

*Plant material.* Suspension cultures of bramble cells were grown under continuous white light at 25° on a synthetic sterile medium as described previously [35]. *N*-Benzyl-8-aza-4$\alpha$,10-dimethyl-*trans*-decal-3$\beta$-ol, 1–10 mg/l., was added in EtOH soln to the culture medium. The drug soln was sterilized by ultrafiltration. Maize (variety LG11) seedlings were allowed to germinate in moist vermiculite for 48 hr at 25°. The seedlings were harvested and the embryos separated from the caryopses.

*Analytical procedure for* in vivo *experiments.* Bramble cells grown in the presence of **7** were harvested by filtration on a nylon cloth (50 $\mu$m); the cells were frozen and then lyophilized. The dried cells (*ca* 2 g) were extracted × 3 with $CH_2Cl_2$. After evaporation of the solvent, the residues was saponified using KOH (6%) in MeOH. The non-saponifiable matter was extracted × 3 with hexane (50 ml). The combined extracts were evaporated under red. pres. and the residue was submitted to TLC with $CH_2Cl_2$ as the solvent (two runs). The isolation of 4,4-dimethyl-, 4$\alpha$-methyl- and 4-desmethylsteryl acetates has been described previously [20–22]. Each of three classes of acetates was analysed by GC with a GC equipped with a FID and a glass capillary column (WCOT, 25 m × 0.25 mm i.d.) coated with OV-1. The temp. program 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% (w/w) $AgNO_3$ impregnated silica gel] in which cyclohexane–toluene (7:3) was the developing solvent and migration was 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 bramble cells and treated cells corresponding, in order of decreasing polarity, to 24-methylene cycloartanyl acetate, 23-acetate and a mixture of $\alpha$- and $\beta$-amyrin acetates. There were three bands of 4$\alpha$-methylsteryl acetates from control bramble cells corresponding, in order of decreasing polarity, to 24-methylenelophenyl acetate, a mixture of **1**- and **2**-acetates and **5**-acetate. There were three bands also for **7**-treated cells. The first band at the same $R_f$ as 24-methylenelophenyl acetate, did not contain this compound but, instead, **10**-acetate. The second band at the same $R_f$ as **1**- and **2**-acetates contained a large amount of **1**-acetate. The third band at the same $R_f$ as **5**-acetate did not contain this compound but instead **4**-acetate. There were three bands of 4-desmethylsteryl acetates from control bramble cells corresponding, in order of decreasing polarity, to 24-methylenecholesteryl acetate, **17**-acetate and a mixture of **18**- and **19**-acetates. From **7**-treated cells there were five bands. The first band at the same $R_f$ as 24-methylenecholesteryl acetate, did not contain this compound but a mixture of **13**-, **14**- and **15**-acetates. The second band contained only **11**- acetate. The third band at the same $R_f$ as **17**-acetate did not contain **17**-acetate but **9**-acetate. The fourth band at the same $R_f$ as **18**- and **19**-acetates, contained only traces of these sterols but enormous amounts of **16**- and **8**-acetates. Finally, the fifth band contained 24-methyl pollinastanol (**29**). All the sterols isolated from **7**-treated cells were identified by their mass and $^1$H NMR spectra as described previously [20–22]. These data, being strictly identical to those already published, have not been detailed here.

*COI and* $\Delta^8 \rightarrow \Delta^7$*-sterol isomerase assays.* Embryos from maize seedlings (60 g) were homogenized with an Ultra-turrax homogenizer in a medium containing 0.1 M Tris–HCl, 5 mM mercaptoethanol and 0.3 M sucrose (pH 7.5). Owing to acidification, the pH was adjusted to 7.5 by addition of Tris–base (1 M). The

homogenate was centrifuged at 6000 *g* for 10 min and the supernatant centrifuged again at 100000 *g* for 1 hr. The pellet (microsomal fraction) was resuspended in a medium (20 ml) containing 0.1 M Tris–HCl and 1 mM mercaptoethanol (pH 7.5). In the case of the COI, the microsomes (0.5 ml ≡ 5 mg protein) were incubated in the presence of cycloeucalenol (**1**) (100 $\mu$M), various concentrations of **7** (10 nM–100 $\mu$M) and Tween-80 [final concn 0.1% (w/v)] at 30° for 45 min. The reaction was stopped by adding 1 ml 6% methanolic KOH. The neutral lipids were extracted with hexane and were analysed by TLC on silica gel using $CH_2Cl_2$ as solvent. The 4,4-dimethyl sterols ($R_f$ 0.45) were separated from 4α-methyl sterols ($R_f$ 0.40) and from 4-desmethyl sterols ($R_f$ 0.30). The 4α-methyl sterols were eluted from the silica gel and analysed by GC equipped with capillary columns as described above. The product of the reaction, obtusifoliol (**2**), was identified and quantified as described previously [4, 24]. In the case of the $\Delta^8 \rightarrow \Delta^7$-sterol isomerase, the microsomes (0.5 ml) were incubated in the presence of **4** (100 $\mu$M), various concns of **7** and Tween-80 [final concn 0.1% (w/v)] at 30° for 45 min–4 hr. Then the reaction mixture was treated as for the COI. The 4α-methyl sterols were analysed by GC. The product of the reaction (**5**) was clearly separated from the substrate (**4**) and was identified by GC/MS as described previously [20].

*Inhibitions assays.* In order to obtain the inhibition curve for each inhibitor and to calculate the $I_{50}$ values, COI and $\Delta^8 \rightarrow \Delta^7$-sterol isomerase assays were conducted with concns of **1** and **4** close to $K_m$ (100 $\mu$M for both **1** and **4**) and at least eight different concns of the same inhibitor. A control where **7** was used as a standard was done in parallel for each inhibitor tested. The $I_{50}$ value obtained for each inhibitor was normalized with respect to the value obtained for **7**.

*Chemical section.* Melting points are uncorr. $^1$H NMR and $^{13}$C NMR spectra were determined in $CDCl_3$ soln operating at 360 MHz with HMDS as int. standard and at 90 and 200 MHz with TMS as int. standard. MS were determined at 70 eV (direct inlet). All NMR data are summarized in Tables 4–7.

*Starting material.* 1-Benzyl-3-methyl-4-piperidone was prepared as described in refs [36, 37]. Yield 69%. bp 0.05 = 110–115°. (Found: C, 76.81–76.70; H, 8.47–8.58; N, 7.10–7.16. $C_{13}H_{17}NO$ requires: C, 76.81; H, 8.42; N, 6.89%.) IR $\nu_{max}^{CHCl_3}$ cm$^{-1}$: 2810, 2770, 1720, 1605, 1590, 1500, 745, 705. $^1$H NMR (60 MHz, $CDCl_3$, TMS): δ0.9 (3H, *d*, *J* = 7 Hz), 1.9–3.1 (7H, *m*), 3.23 (2H, *s*), 7.1 (5H, *s*). MS *m/z*: 203, 202, 146, 126, 112, 91, 42.

*N-Benzyl-8-aza-4,10-dimethyl-decal-4-en-3-one* (**30**). *N*-Benzyl-3-methyl-4-piperidone (193 g) dissolved in 200 ml MeOH was added at −5° to a soln of NaOMe (56 g) in MeOH (2 l.). After 1 hr at −5°, ethylvinylketone (120 g) dissolved in 200 ml MeOH was added at −5° and the reaction mixture stirred for 4 hr at −5°, then for 15 hr at 20°. After acid hydrolysis, MeOH was evaporated, the reaction mixture poured on an iced-cold $Na_2CO_3$ soln and then extracted with $Et_2O$. After evaporation of the ethereal soln, the crude product (283 g) of the reaction

Table 4. $^1$H NMR ($CDCl_3$, HMDS) chemical shifts (δ) of the proton signals of various 8-aza-decalins

| Compound | 1a* | 1e* | 2a | 2e | 3a | 4a | 4e† | 5a | 6a | 6e | 7a | 7e | 9a | 9e | 10a† | 11 (AB)‡ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **30** | 1.6 | 1.48 | 2.4 | 2.33 | — | — | 1.68 | — | 2.4 | 2.58 | 1.95 | 2.96 | 1.72 | 2.51 | 1.29 | 3.51–3.33 |
| **31** | 1.36 | 1.5 | 2.44 | 2.26 | — | 2.18 | 0.90 | 1.02 | 1.5 | —§ | 1.84 | 2.90 | 1.52 | 2.48 | 1.19 | 3.47–3.30 |
| **7** | 1.04 | 1.12 | 1.5 | 1.73 | 3.04 | 1.22 | 0.88 | —§ | 1.28 | 1.15 | 1.82 | 2.89 | 1.44 | 2.38 | 0.98 | 3.45–3.28 |
| **20** | 1.05 | 1.22 | 1.53 | 1.77 | 3.04 | 1.19 | 0.87 | 1.02 | 1.11 | 1.47 | 2.47 | 3.08 | 2.19 | 2.53 | 0.89 | — |
| **25** | 1.02 | 1.2 | 1.5 | 1.71 | 3.03 | 1.2 | 0.87 | 0.61 | 1.28 | 1.15 | 1.87 | 2.85 | 1.42 | 2.32 | 0.95 | 3.38–3.22 |
| **26** | 1.04 | 1.2 | 1.52 | 1.73 | 3.04 | —§ | 0.88 | 0.64 | 1.32 | 1.5 | 1.87 | 2.87 | 1.46 | 2.33 | 0.98 | 3.48–3.31 |
| **27** | —§ | —§ | 1.5 | 1.74 | 3.04 | —§ | 0.88 | 0.60 | —§ | 1.5 | 1.71 | 2.87 | 1.34 | 2.44 | 0.93 | 2.20–2.06 |
| **32** | 1.38 | 1.47 | 2.53 | 2.17 | — | 0.90† | 0.88 | 1.11 | 1.59 | 1.29 | 1.78 | 2.86 | 1.43 | 2.30 | 1.09 | 3.38–3.19 |
| **28** | —§ | —§ | —§ | 1.58 | 3.18 | 0.96† | 0.89 | —§ | 1.58 | 1.43 | 1.85 | 2.94 | 1.43 | 2.27 | 1.0 | 3.15–3.31 |

*a, Axial; e, equatorial.
†Methyl.
‡The signals for phenyl hydrogen are not described.
§Masked signal.

Table 5. Coupling constants (Hz) for hydroxy compounds: **7**, **20**, **25**, **26** and **28**

| 9e | 7a | 7e | 6a | 6e | 5 | 4a | 3a | 2a | 2e | 1a | 1e | H |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| −10.5 | | | | | | | | | | | | 9a |
| | | 2 | | | | | | | | | | 9e |
| | | −11 | 12 | 3 | | | | | | | | 7a |
| | | | 4 | 12 | | | | | | | | 7e |
| | | | | −12 | 12 | | | | | | | 6a |
| | | | | | 3.5 | | | | | | | 6e |
| | | | | | | 11 | | | | | | 5 |
| | | | | | | | 11 | | | | | 4a |
| | | | | | | | | 10 | 5 | | | 3a |
| | | | | | | | | | −12 | 13.5 | | 2a |
| | | | | | | | | | | 4 | | 2e |
| | | | | | | | | | | | −13.5 | 1a |

Table 6. Coupling constants ($H_2$) for ketones **31** and **32**

| 9e | 7a | 7e | 6a | 6e | 5 | 4a | 2a | 2e | 1a | 1e | H |
|---|---|---|---|---|---|---|---|---|---|---|---|
| −10.5 | | | | | | | | | | | 9a |
| | | 2 | | | | | | | | | 9e |
| | | −11 | 12 | 3 | | | | | | | 7a |
| | | | 4 | 3 | | | | | | | 7e |
| | | | | −12 | 12 | | | | | | 6a |
| | | | | | 3 | | | | | | 6e |
| | | | | | | 12 | | | | | 5 |
| | | | | | | | | −16 | 11 | 5.5 | 2a |
| | | | | | | | | | 5.0 | 3 | 2e |
| | | | | | | | | | | −13 | 1a |

Table 7. $^{13}C$ NMR (CDCl, TMS) chemical shifts ($\delta$) of aza-decalins **7**, **30**, **31** and **32**

| Carbon No. | **30** | **31** | **7** | **32** |
|---|---|---|---|---|
| C-1 | 34.4 | 38.4 | 36.7 | 36.3 |
| C-2 | 33.5 | 37.7 | 30.8 | 27.4 |
| C-3 | 198.5 | 212.2 | 77.0 | 79.4 |
| C-4 | 128.6 | 45.1 | 39.6 | 38.4 |
| C-5 | 159.7 | 50.2 | 48.2 | 51.8 |
| C-6 | 28.1 | 26.5 | 25.3 | 22.1 |
| C-7 | 53.9 | 54.5 | 55.3 | 55.7 |
| C-9 | 66.9 | 66.7 | 67.6 | 70.6 |
| C-10 | 37.2 | 34.5 | 34.4 | 34.3 |
| $CH_3$-10 | 23.1 | 17.2 | 17.9 | 20.0 |
| $CH_3$-4e* | 10.5 | 10.9 | 14.7 | 27.6 |
| $CH_3$-4a* | — | — | — | 15.1 |
| C-11† | — | 62.9§ | | — |
| *ipso*‡ | — | 139.2§ | | — |
| *ortho*‡ | — | 128.1§ | | — |
| *meta*‡ | — | 128.7§ | | — |
| *para*‡ | — | 126.8§ | | — |

*a, Axial.
†e, equatorial.
‡benzylic (= phenyl).
§Average.

was distilled under red. pres. bp 0.05 = 170° to give 198 g (yield 77.5%) of **30** as a pale yellow solid, mp 67° (crystals from hexane). (Found: C, 80.39–80.40; H, 8.52–6.67; N, 5.10–5.12; O, 6.10–6.11. $C_{18}H_{23}NO$ requires: C, 80.25; H, 8.60; N, 5.19; O, 5.93.) IR $\nu_{max}^{KBr}$ $cm^{-1}$: 2810, 2780, 1670, 1610, 745, 700. MS *m/z*: 269, 254, 226, 192, 178, 91.

N-*Benzyl*-8-*aza*-4α,10-*dimethyl*-trans-*decal*-3-*one* (**31**). A soln of ketone **30** (27.0 g) and dry *t*-BuOH (7.04 g) in 400 ml dry THF was added within 40 min at −30° to a soln of Li (2.1 g) in 2 l. dry $NH_3$ (distilled on Li). After stirring for 30 min, the excess of Li was destroyed by adding bromobenzene until the reaction mixture became clear, then satd $NH_4Cl$ soln was added, $NH_3$ was evaporated and the mixture extracted with $Et_2O$. The organic phase was washed with $H_2O$, dried over $Na_2SO_4$ and concd to give 28.0 g (yield 100%) of **31** as a yellow oil. IR $\nu_{max}$ $cm^{-1}$: 2920, 1710, 735, 700. MS *m/z*: 271, 256, 180, 147, 146, 134, 120, 91.

N-*Benzyl*-8-*aza*-4α,10-*dimethyl*-trans-*decal*-3β-*ol* (**7**). A soln of crude ketone **31** (28 g) in 500 ml $Et_2O$ was added at 0° to $LiAlH_4$ (5.70 g) in 1 l. of dry $Et_2O$, and the mixture was reacted for 1 hr at 5° then for 1 hr at 20°. Usual work-up gave 27.3 g yellow oil which after crystallization from hexane, afforded 16.8 g (yield 60%) of pure 3β-OH **7**: mp 68°. (Found: C, 79.12–79.37; H, 9.82–10.20; N, 5.02. $C_{18}H_{27}NO$ requires: C, 79.07; H, 9.95; N, 5.12; O, 5.85%.) IR $\nu_{max}^{CCl_4}$ $cm^{-1}$: 3620, 3500, 3300, 1020, 1010, 745, 705. MS *m/z*: 273, 256, 255, 182, 147, 146, 134, 120, 91.

8-*Aza*-4α,10-*dimethyl*-trans-*decal*-3β-*ol* (**20**). The *N*-benzyl-decalin (**7**) was dissolved in 600 ml glacial HOAc, 3.0 g 5% Pd/C was added and the mixture was stirred at 25° under $H_2$ at atm pres. until $H_2$ uptake ceased (2.5 hr). The catalyst was removed by filtration and washed with $Et_2O$ and EtOAc. The soln was concd to dryness under red. pres. and 50 ml $H_2O$ and then solid $Na_2CO_3$ were added until the pH reached 10. The $H_2O$ was evaporated and the solid residue extracted with $CHCl_3$ under reflux for 30 min. Filtration of the hot soln and evaporation of $CHCl_3$ afforded 10.9 g of an oil which, after chromatography on silica gel with MeOH–$NH_4OH$ (92:8) gave 8.9 g (yield 88.7%) of white solid (**20**): mp 130°. IR $\nu_{max}^{KBr}$ $cm^{-1}$: 3400, 3320, 3220, 1050. MS *m/z*: 183, 168, 165, 150, 57, 44, 30.

N-4-*Chlorobenzyl*-4α,10-*dimethyl*-trans-*decal*-3β-*ol* (**25**). A mixture of **20** (1.2 g), α-*p*-dichlorotoluene (1.11 *g*) and $K_2CO_3$ (0.905 g) in 15 ml MeCN was refluxed for 5.25 hr. The solvent was evaporated, 50 ml $H_2O$ added and the mixture extracted with $Et_2O$. The organic soln was washed with a satd NaCl soln, dried over $Na_2SO_4$ and evaporated to give 2.25 g white solid which, after chromatography on silica gel with hexane–EtOAc (80:20) afforded 1.83 g (yield 90.7%) **25**: mp 94° (crystals from hexane). (Found: C, 70.15–70.29; H, 8.43–8.62; Cl, 11.39–11.42; N, 4.39–4.59; O, 5.27–5.31. $C_{18}H_{26}ClNO$: C, 70.22; H, 8.51; Cl, 11.51; N, 4.55; O, 5.20%.) IR $\nu_{max}^{KBr}$ $cm^{-1}$: 3260, 1025, 1010, 800. MS *m/z*: 307, 306, 290, 274, 180, 168, 125. Similarly the following compounds were synthesized: **24**, mp 90–92° (yield 74%); **26**, oil (yield 85.4%); **27**, mp 59% (yield 65.5%).

N-*Benzyl*-8-*aza*-4,4,10-*trimethyl*-trans-*decal*-3-*one* (**32**). A soln of the ketone **30** (5.4 g) in 100 ml dry dioxane was added at −30° within 1.25 hr to a soln of Li (0.56 g) in 400 ml dry $NH_3$. After stirring for 2.5 hr at −30°, the excess of Li was destroyed by adding bromobenzene until the reaction mixture became clear. $NH_3$ was evaporated and successively were added 50 ml dioxane and 50 ml MeI. After stirring under reflux for 1 hr, the reaction mixture was evaporated and 100 ml $H_2O$ then added. Extraction with $CH_2Cl_2$, evaporation of the solvent and chromatography on silica gel with hexane–EtOAc (9:1) afforded 0.87 g (15%) of the ketone **32** as a pale yellow oil. IR $\nu_{max}$ $cm^{-1}$: 1710, 740, 695. MS *m/z*: 285, 270, 194, 188, 146, 91.

N-*Benzyl*-8-*aza*-4,4,10-*trimethyl*-trans-*decal*-3β-*ol* (**28**). The ketone **32** (1.60 g) was reduced with $LiAlH_4$ in $Et_2O$ as above. Chromatography of the crude product with hexane–EtOAc (80:20) afforded 1.41 g (87.7%) of **28**: mp 92°. IR $\nu_{max}^{KBr}$ $cm^{-1}$:

2240, 1365, 1030, 1015, 735, 695. MS *m/z*: 287, 272, 270, 196, 147, 146, 134, 91.

4α,10-*Dimethyl*-trans-*decal*-3β-*ol* (**20**). This compound was synthesized according to ref. [38].

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