

In vitro Incorporation of ^2H at the C-10 Methyl Group of Obtusifoliol during the Enzymatic Cleavage of the $9\beta,19\beta$ -Cyclopropane Ring of Cycloeucalenol

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Summary Incubation of cycloeucalenol with microsomes of *Zea mays* embryos in D_2O yields $[19-^2\text{H}]$ obtusifoliol.

$9\beta,19\beta$ -CYCLOPROPYL sterols, cycloartenol (1), 24-methylene cycloartanol (2), and cycloeucalenol (3) are considered to be intermediates in sterol biosynthesis in photosynthetic eukaryotes.¹ When germinating peas are grown in D_2O , $[19-^2\text{H}]$ sitosterol is biosynthesized,² suggesting that ^2H incorporation occurs during $9\beta,19\beta$ -cyclopropane ring opening of $9\beta,19\beta$ -cyclopropyl sterols, an obligatory step in phytosterol biosynthesis. We have shown previously³ that opening of the cyclopropane ring occurs when (3) is incubated in the presence of higher plant microsomes giving obtusifoliol (4), another presumed intermediate in phytosterol biosynthesis.¹ Under the same conditions (1) was

not converted into lanosterol (5). We report here evidence that the ^2H incorporation at C-19 described earlier² occurs during enzymic conversion of (3) into (4).

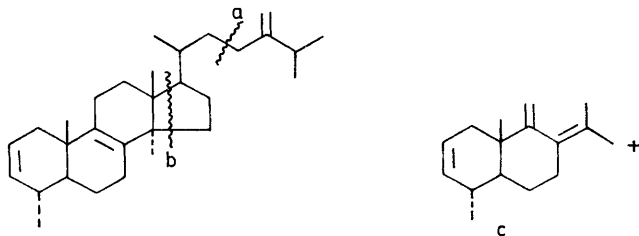
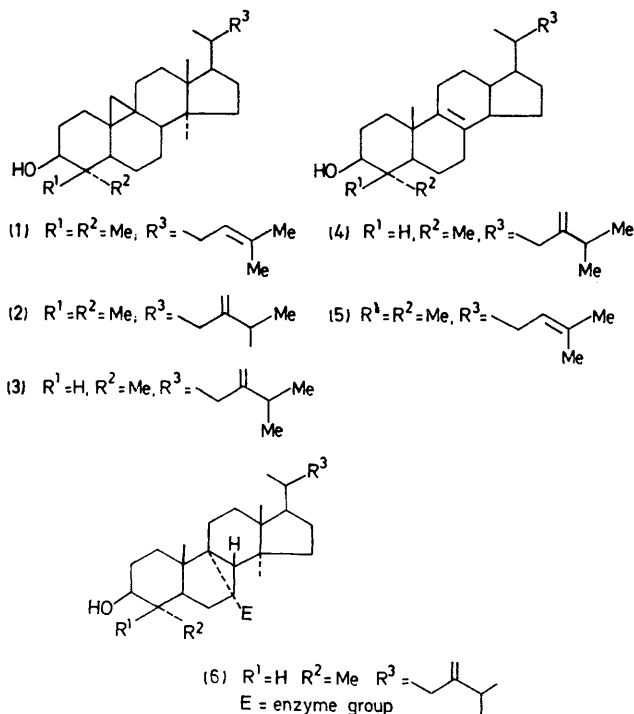
Microsomes from maize embryos were prepared as described earlier.³ They were washed with D_2O with phosphate buffer (pH 8.3), containing mercaptoethanol (5 mM) and MgCl_2 (2 mM). After centrifugation ($100,000 \times g$, 60 min), the pellets were resuspended in the same medium and incubated (30 °C; 12 h) in the presence of cycloeucalenol (3) ($150 \mu\text{M}$) emulsified with Tween 80 (0.1% in the incubation). Extraction of sterols and chromatography were performed as described.³ The 4α -methyl sterols were analysed by g.l.c.-mass spectroscopy as their Me_3Si ethers. Under these conditions, the substrate (3) was unambiguously separated from the product (4) of the reaction and mass spectra were recorded. The average yield for the conversion of (3) into (4) was >70%. The mass spectra showed a 66% incorporation of ^2H in the molecular ion of the Me_3Si ether of (4) (Table). No incorporation of ^2H was detected in charged (3). The ^2H label was retained in fragments^{4,5} a, b, and c (Table), in agreement with the hypothesis of the location of ^2H at C-19.

TABLE

Mass spectral determination of ^2H incorporation in Me_3Si ether of (4) and in fragments⁴ (average of three experiments).

| m/e | 498 | 483 | 393 | 309 | 227 | 215 |
|---|-------|------------|---|-----|-----|-----|
| | M^+ | $M^+ - 15$ | $M^+ - \text{Si}(\text{Me})_2 - \text{OH} - 15$ | a | b | c |
| ^2H incorporation ^a % | 66 | 65 | 60 | 59 | 55 | 55 |

^a estimated from $m/e + 1$ peaks; standard deviation $\pm 2\%$.



To prove this point, the n.m.r. (90 MHz) spectra of the mixture of (3) (30%) and (4) (70%) were recorded in the presence of increasing amounts (0.0—1.4 molar ratio) of the shift reagent $\text{Eu}(\text{fod})_3$. At ca 1:1 molar ratio, the 4α - and 10-methyl-resonances are strongly shifted downfield^{6,7} and clearly separated from other signals. Comparison of the spectra of pure (4) and of the deuteriated product shows a decrease in the height and a broadening of the 10-Me signal in the deuteriated product. The broadening is caused by differences in the chemical shifts for CH_3 and CH_2D , and by the CH_2D triplet.⁸ Moreover, integration indicated an incorporation of 0.6 ± 0.1 of ^2H , using the 4α -methyl-resonance of obtusifoliol as internal standard, thus confirming that the ^2H incorporation into (4) detected by mass spectroscopy occurs essentially at the 10-methyl-group. These results allow us to conclude that during transformation of cycloecalenol into obtusifoliol in D_2O , ^2H is incorporated in the 10-methyl group, and suggest that this enzymic reaction could be considered as a biochemical step equivalent to an acid-catalysed cyclopropane ring opening reaction⁹ in agreement with the mechanism previously proposed.⁴

Cycloartenol (1) was incubated with microsomes from maize embryos in D_2O (see above). As expected,³ (1) was not converted into lanosterol (5). Moreover, recovered (1) had not incorporated ^2H , as was also the case for unchanged (3). It has been suggested that the conversion of (3) into (4) involves an intermediate (6) in which the C-9 carbonium ion is stabilised (ionic pair or covalent bond) by a suitable enzymatic group; (4) is obtained *via* a *trans* antiperiplanar elimination of $8\beta\text{-H}$ from (6). No incorporation of ^2H was shown to occur into (3) during its conversion into (4); it follows that the transformation of (3) into (6) proceeds without exchange of the 19-proton with protons from the medium and so this latter step could be considered to be irreversible. This interpretation, however, implies that the 10-methyl group in (6) retained its free rotation.

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¹ L. J. Goad and T. W. Goodwin, in 'Progress in Phytochemistry,' eds. L. Rheinhold and Y. Liwischitz, Interscience, London, 1972, vol. III, p. 113.

² E. Caspi and J. Sliwowski, *J. Amer. Chem. Soc.*, 1975, **97**, 5032.

³ R. Heintz and P. Benveniste, *J. Biol. Chem.*, 1974, **249**, 4267.

⁴ L. J. Goad, B. L. Williams, and T. W. Goodwin, *European J. Biochem.*, 1967, **3**, 232.

⁵ H. Budzikiewicz, J. M. Wilson, and C. Djerassi, *J. Amer. Chem. Soc.*, 1963, **85**, 3688.

⁶ M. Kocor and J. St. Pyrek, *J. Org. Chem.*, 1973, **38**, 3688.

⁷ T. Suga and T. Shishibori, *Phytochemistry*, 1975, **14**, 2411.

⁸ C. G. Macdonald, J. S. Shannon, and S. Sternhell, *Austral. J. Chem.*, 1964, **17**, 38.

⁹ H. R. Bentley, J. A. Henry, D. S. Irvine, and F. S. Spring, *J. Chem. Soc.*, 1953, 3673.