$C_{32}H_{52}O_2$: C, 82.05; H, 11.11%.) Oxidation of **ii** with a CrO_3–Py complex [12] gave a ketone, mp 180–182°, IR ν_{max}^{Nujol} cm^{-1}: 1710, 845. (Found: C, 84.86; H, 11.50. Calc. for $C_{30}H_{48}O$: C, 84.9; H, 11.32%.)

Acknowledgement—We are greatly indebted to Professor R. C. Cookson F.R.S., of the University of Southampton, U.K., for MS.

REFERENCES

1. Ayyar, K. S., Shankara narayana, K. H., Sivaramakrishnan, V. R. and Sen Sarma, P. K. (1977) Proceedings of the All India Sandal Seminar, p. 120. Bangalore, India (7 February).
2. Williams, C. M., (1967) *Sci. Am.* **217,** 13.
3. Meisels, V. R., Jeger, O. and Ruzicka, L. (1950) *Helv. Chim. Acta.* **33,** 700.
4. Cole, A. R. H., (1954) *J. Chem. Soc.* 3807.
5. Parmer, M. A., and Bowden, B. N. (1975) *Phytochemistry* **14,** 2049.
6. Djerrassi, C., Budzikiewicz, H. and Wilson, J. M. (1962) *Tetrahedron Letters* 263.
7. Karlinger, J. and Djerrassi, C. (1966). *J. Org. Chem.* **31,** 1945.
8. Cheung, H. T. and Yan, T. C. (1972) *Aust. J. Chem.* **25,** 2003.
9. Appelton, R. A. and Enzell, C. R. (1971) *Phytochemistry* **10,** 447.
10. Bryce, T. A., Eglinton, G., Hamilton, R. J., Smith, M. M. and Subramaniyan, G. (1967) *Phytochemistry* **6,** 627.
11. Atallah, A. M. and Nicholas, H. J. (1971) *Phytochemistry* **10,** 3139.
12. Ratcliffe, R. and Rodehorst, R. (1970) *J. Org. Chem.* **35,** 4000.

Phytochemistry, 1980, Vol. 19, pp. 1240–1242. 0031-9422/80/0601-1240 $02.00/0

LANOSTEROL, THE FIRST POLYCYCLIC INTERMEDIATE IN STEROL BIOSYNTHESIS BY *UROMYCES PHASEOLI**

SURENDRA K. BANSAL† and HERMAN W. KNOCHE

Laboratory of Agricultural Biochemistry, University of Nebraska, Lincoln, NE 68583, U.S.A.

(*Received* 31 *July* 1979)

Key Word Index—*Uromyces phaseoli*; bean rust; fungal sterols; sterol biosynthesis; first polycyclic intermediate; squalene; lanosterol; cycloartenol.

INTRODUCTION

Dichotomy at the point of cyclization of squalene has been well established in sterol biosynthesis; lanosterol is the first polycyclic compound in the biosynthesis of sterols in animals while cycloartenol is found in photosynthetic plants and algae [1, 2]. Fungi have been considered to follow the pathway operating in animals. Support for this conclusion came from the isolation of lanosterol from *Saccharomyces cerevisiae* [3], *Phycomyces blakesleeanus* [4] and *Aspergillus fumigatus* [4] and from the demonstration of 2,3-oxidosqualene-lanosterol cyclase in cell-free preparations of *Saccharomyces cerevisiae* [5] and *Phycomyces blakesleeanus* [6]. Furthermore, other lanostane-type structures have been found in some of the wood-rotting fungi [7] but cycloartenol has not been detected in any fungi whose sterol contents have been determined [7, 8]. The conclusion that lanosterol is the first polycyclic precursor in fungi is based on a generalization drawn from the three species mentioned above which contain lanosterol in detectable amounts.

It has been observed previously that photosynthetic organisms synthesize cycloartenol, yet both lanosterol and cycloartneol are utilized equally well in the synthesis of 4-desmethyl sterols [9]. However, in rat liver, cycloartenol is not metabolized to 4-desmethyl sterols whereas lanosterol is utilized [9]. To our knowledge, lanosterol and cycloartenol utilization studies have not been carried out for any fungal species. Thus the purpose of this work was to determine the first polycyclic product in sterol biosynthesis for *Uromyces phaseoli*, which is an obligately parasitic fungus that differs taxonomically from the fungi mentioned above. Cycloartenol and lanosterol have not been detected among the sterols of this organism [10]. A second

* Published with the approval of the Director as paper No. 5829 Journal series, Nebraska Agricultural Experiment Station. Research reported was conducted under project 15-018. This study was supported by the Nebraska Wheat Division.

† Present address: Department of Experimental Therapeutics, Roswell Park Memorial Institute, Buffalo, NY 14263, U.S.A.

objective was to determine if either of these sterols were preferentially utilized by the fungus.

RESULTS AND DISCUSSION

Trapping of radioactivity in the first polycyclic compound

Lanosterol or cycloartenol (4 mg) and squalene-[^{14}C] (2 mg, 1.06×10^6 dpm) were emulsified with 24 mg Tween-80, and 5 μmol NADPH, dissolved in 1 ml 0.01 M Tris–HCl buffer, pH 7.5, were added to the emulsion. The mixtures were preincubated at 23° for 5 min followed by addition of 4 ml of a 10 000 g supernatant of the homogenate of uredospores of *Uromyces phaseoli*. After 2 hr at 23° the reaction was stopped by adding KOH and EtOH to final concentrations of 10 and 50%, respectively. The incubation mixtures were saponified and the unsaponifiable materials were subjected to TLC with Et_2O–hexane–HOAc (70 : 30 : 1) as the solvent. The zones cochromatographing with authentic lanosterol or cycloartenol (identical mobilities) were removed and extracted with $CHCl_3$. The concentrated $CHCl_3$ extracts were rechromatographed (TLC) with cyclohexane–EtOAc (90 : 10) as the solvent. Again the zones cochromatographing with lanosterol or cycloartenol were removed and extracted with $CHCl_3$. To the material isolated from the lanosterol-trap experiment, 6 mg non-radioactive lanosterol was added and to the corresponding extract from the cycloartenol-trap experiment an equal amount of non-radioactive cycloartenol was added. After evaporating the $CHCl_3$, the residues were acetylated and the steryl acetates were chromatographed on $AgNO_3$–Si gel TLC with $CHCl_3$–HOAc (200 : 1) as the solvent. The zones corresponding to lanosteryl acetate or cycloartenyl acetate (identical mobilities) were removed and extracted with $CHCl_3$. To the material isolated from the lanosterol-trap experiment, 10 mg non-radioactive lanosteryl acetate was added and to the extract from the cycloartenol-trap experiment an equal amount of non-radioactive cycloartenyl acetate was added. Solvents were evaporated and the residues were dried overnight *in vacuo*. The steryl acetates were then recrystallized from MeOH–H_2O and their specific radioactivities were determined (Table 1). The purities of the steryl acetates were confirmed by mp and GLC. In two other similar experiments the initial specific radioactivities were lower but again the specific radioactivity of lanosteryl acetate became constant and that of cycloartenyl acetate dropped to zero within five recrystallizations.

Preferential utilization of lanosterol

The 1500 *g* supernatant of the homogenate of uredospores of *U. phaseoli* was incubated with a mixture of lanosterol and cycloartenol. The incubation mixture contained Tris–HCl buffer, pH 7.5 (0.01 M), ATP (3 mM), NADPH (1 mM), glucose 6-phosphate (3 mM), nicotinamide (30 mM), $MgCl_2$ (5 mM), $MnCl_2$ (2 mM) and 0.5 mg each of lanosterol and cycloartenol. The sterols were suspended in the aqueous media with the aid of the detergent Lubrol PX (32 mg). Total incubation volume was 8 ml after the addition of 5 ml of 1500 *g* supernatant of the homogenate.

Table 1. Radioactivity associated with lanosterol and cycloartenol after incubation of Uromcyes phaseoli homogenate with squalene-[^{14}C]

Cold trap	Lanosterol	Cycloartenol
Initial weight	18.85 mg	19.50 mg
	Specific radioactivities of acetates (dpm/mg)	
Before recrystallization	7.62 ± 0.26	4.48 ± 0.25
After 3 recrystallizations	7.51 ± 0.27	2.21 ± 0.25
After 5 recrystallizations	8.07 ± 0.26	0.45 ± 0.25
After 6 recrystallizations	7.97 ± 0.26	0.00 ± 0.25
Melting point	129–130°	123–124°
Literature value	129–130°*	122–124°†

* Melting point of lanosteryl acetate [13].
† Melting point of cycloartenyl acetate [14].

Incubation was carried out for 3 hr and at the end of the first and second hour 24 μmol ATP, 8 μmol NAD and 48 μmol EtOH dissolved in 0.1 ml 0.01 M Tris–HCl buffer, (pH 7.5) were added [11]. The reaction was stopped by adding 8 ml 2 N KOH in 50% EtOH. A blank was prepared in an identical manner except that the reaction was stopped at zero time. The unreacted lanosterol and cycloartenol were then isolated from the unsaponifiable materials by TLC (Et_2O–heptane–HOAc, 70 : 30 : 1). The zones cochromatographing with lanosterol or cycloartenol were removed, extracted with $CHCl_3$ and acetylated. Acetylated products were chromatographed on $AgNO_3$–Si gel TLC with $CHCl_3$–HOAc (200 : 1). Appropriate zones were removed, extracted with $CHCl_3$, concentrated and analysed by GLC. Lanosteryl acetate and cycloartenyl acetate had retention times of 17.6 and 20.8 min, respectively. The relative concentrations of the sterols were determined by calculating the peak areas by triangulation. For the zero-time-blank a lanosterol : cycloartenol ratio of 1.01 was obtained and for the 3 hr incubation the ratio was 0.548.

Other experiments with slightly different homogenization procedures yielded similar results, that is, an appreciable decrease in lanosterol relative to cycloartenol in the unmetabolized sterol pool was observed. Thus lanosterol was preferentially utilized by the fungus, and it is assumed that it was used mainly for 4-demethyl sterol productions.

The data presented provide evidence that lanosterol is the first polycyclic product in the biosynthesis of sterols in *U. phaseoli*, a Basidiomycete. Thus the results support the hypothesis that lanosterol is the first polycyclic compound in the biosynthesis of sterols in diverse classes of fungi. The preferential utilization of lanosterol by the fungus shows that it may be closer to animals in respect to 4,4-dimethylsterol metalbolism, since animals can utilize only lanosterol, whereas photosynthetic organisms are able to utilize both lanosterol and cycloartenol to synthesize their dominant sterols.

EXPERIMENTAL

Bean rust uredospores, *Uromyces phaseoli* (pers.) Wint. var., typica Arth., were produced as described by Trocha and Daly [12].

Homogenization of uredospores. Uredospores (1 g) were homogenized for 1 min (MSK homogenizer, 4000 oscillations per min) in a 50 ml bottle containing 5 g glass beads (1 mm dia) and 5 ml 0.01 M Tris–HCl buffer, pH 7.5. During homogenization the bottle was cooled by liquid CO_2. The homogenate and glass beads were washed with 1 ml buffer and the wash was added to the homogenate, which was centrifuged at 1500 g for 20 min. In some experiments this supernatant was used while for others this supernatant was centrifuged at 10 000 g for 30 min and the resulting supernatant was used for incubations.

Chromatography. TLC was performed with 0.75 mm thick Si gel G or 15% $AgNO_3$-Si gel plates. The bands were detected by spraying with Rhodamine 6G and viewing under UV light. GLC was performed with stainless steel column, (2 m × 3 mm) packed with 1.5% OV-17 operated at 270°; the carrier gas was N_2 at 60 ml/min.

Radioactive assay. Samples were dissolved in 10 ml of a soln of PPO in toluene (4 g/l). The samples along with background blanks were counted to give 98% statistical accuracy. The efficiencies were determined by the external standard channel ratio method.

Lanosterol. Commercial lanosterol (70–80% pure, Sigma Chemical Co.) was purified by acetylating and chromatographing the products on $AgNO_3$-Si gel TLC with $CHCl_3$–HOAc (200 : 1). The lanosteryl acetate was saponified and the resulting lanosterol was recrystallized (2×) from MeOH–$CHCl_3$, mp 137.5–138° (lit. [13] 140°).

Cycloartenol. Cycloartenol was isolated from seeds of *Strychnos nux-vomica* [14], which were a gift from Penick, Inc., Lyndhurst, NJ, U.S.A. Seeds (600 g) were ground in a mill and extracted with $CHCl_3$ in a Soxhlet apparatus for 48 hr which yielded 52 g fat. The fat was saponified by refluxing for 2 hr with 500 ml 2 N KOH in EtOH and the non-saponifiables, extracted with petrol–Et_2O (1 : 1), were dissolved in petrol–Et_2O (3 : 1) and percolated through a column (1.5 cm internal dia) of 95 g Al_2O_3 (Brockman grade II/III). The column was eluted with 2.5 l. of petrol–Et_2O (3 : 1)(250 ml/hr) and 150 ml fractions were collected. The cycloartenol containing fractions (TLC) were pooled and acetylated, and then purified on $AgNO_3$-Si gel TLC ($CHCl_3$–HOAc, 200 : 1). The cycloartenyl acetate (400 mg) was eluted with $CHCl_3$ and was then recrystallized from MeOH–$CHCl_3$, mp 123.5–124°, lit. [14] 122–124; saponification of the acetate followed by recrystallization (2×) from MeOH gave cycloartenol. The mp of the recrystallized product after overnight drying *in vacuo* at 65° was 115° sharp, lit. [14] 115°. The purity of the isolated cycloartenol was found to be more than 98% on GLC. The standard cycloartenyl acetate used in the GLC and TLC was obtained as a gift from the Steriod Reference Collection, Chemistry Department, Westfield College, Hampstead, London; through Nat. Inst. of Health, Bethesda, MD, U.S.A.

Squalene-[^{14}C]. Squalene-[^{14}C] was synthesized by incubating, anaerobically, DL-mevalonic acid-[2-^{14}C] with a S_{10} preparation of pig liver, as described by Popjak [11]. The squalene-[^{14}C] isolated from the incubation mixture was purified on an Al_2O_3 column [11].

REFERENCES

1. Goad, L. J. and Goodwin, T. W. (1972) in *Progress in Phytochemistry* (Reinhold, L. and Liwschitz, Y., eds.), Vol. 3, p. 113. Interscience, New York.
2. Nes, W. R. (1977) in *Advances in Lipid Research* (Paoletti, R. and Kritchevsky, D., eds.) p. 233. Academic Press, New York.
3. Wieland, H., Pasedach, H. and Balluf, A. (1937) *Justus Liebigs Ann. Chem.* **529,** 68.
4. Goulston, G., Goad, L. J. and Goodwin, T. W. (1967) *Biochem. J.* **102,** 15C.
5. Schechter, I., Sweat, F. W. and Bloch, K. (1970) *Biochim. Biophys. Acta* **110,** 463.
6. Mercer, E. I. and Johnson, M. W. (1969) *Phytochemistry* **8,** 2329.
7. Yokoyama, A., Natori, S. and Aoshima K. (1975) *Phytochemistry* **14,** 487.
8. Weete, J. D. (1973) *Phytochemistry* **12,** 1843.
9. Gibbons, G. F., Goad, L. J., Goodwin, T. W. and Nes, W. R. (1971) *J. Biol. Chem.* **246,** 3967.
10. Lin, H. K., Langenback, R. J. and Knoche, H. W. (1972) *Phytochemistry* **11,** 2319.
11. Popjak, G. (1969) in *Methods in Enzymology* (Clayton, R. B., ed.) p. 393. Academic Press, New York.
12. Trocha, P. and Daly, J. M. (1972) *Plant Physiol.* **46,** 520.
13. Lewis, D. A. and McGhie, J. F. (1956) *Chem. Ind. (London)* 550.
14. Bentley, H. R., Henry, J. A., Irvine, D. S. and Spring, F. S. (1953) *J. Chem. Soc. (London)* 3673.