Phytochemistry, 1975, Vol. 14, pp. 727–730. Pergamon Press. Printed in England.

STEROL BIOSYNTHESIS IN HETEROTROPHIC PLANT PARASITES

M. ROHMER,* G. OURISSON,† P. BENVENISTE* and T. BIMPSON‡

* Institut de Botanique, Laboratoire de Biochimie Végétale, 28 rue Goethe, 67083 Strasbourg-Cedex, France; † Institut de Chimie, Laboratoire de Chimie Organique des Substances Naturelles, 1 rue Blaise Pascal, 67008 Strasbourg-Cedex, France;‡ "Cherrywood", 42, Well Lane, Weaverham, Northwich, Cheshire, England

(*Received* 14 *September* 1974)

Key Word Index—*Cuscuta europaea*; *C. epithymum*; *Convolvulaceae*; *Orobanche lutea*; Orobanchaceae; sterol biosynthesis; cycloartenol; plant parasites.

Abstract—Cycloartenol derivatives are present in the non-photosynthetic parasitic plants *Cuscuta europaea* (dodder), *Cuscuta epithymum* and *Orobanche lutea* (broomrape). *C. europaea* and *O. lutea* are capable of biosynthesizing their own sterols. There is therefore no direct link, in a chlorophyll-containing phylum, between the cycloartenol pathway to sterols and photosynthesis.

INTRODUCTION

It is now generally accepted that a dichotomy exists in sterol biosynthesis: lanosterol is the triterpene intermediate in animals and fungi (yeast) and cycloartenol in higher plants [1]. The suggestion that the presence of cycloartenol is intimately connected with the presence or absence of the photosynthetic apparatus in the tissue was not confirmed by studies with *Euglena gracilis* [2], *Astasia longa* [3], a natural white mutant of *Euglena*, and with the endosperm of pine seeds (*Pinus pinea*) [4]. However, we considered that this problem was not fully resolved, and we therefore decided to study heterotrophic plant parasites. These organisms, in contrast to the pine endosperm which represents a developmental step of the life cycle of this plant, are not (*Orobanche sp.*) or only partly (*Cuscuta sp.*) photosynthetic during their life cycles.

We intended to answer the following questions: are these organisms heterotrophic for sterols and utilize sterols produced by the host or are they capable of synthesizing their own sterols; and, in the latter case, what is the nature of the triterpene intermediate?

RESULTS

Incorporations and analyses were carried out in duplicate with identical results. The weight of plants extracted and the amounts of products isolated were: *O. lutea* 16 g dry wt 500 μg 4,4-dimethylsterols, 500 μg 4α-methylsterols, 12 mg other sterols; and *C. epithymum* 23 g dry wt, 150 μg 4,4-dimethylsterols, 450 μg 4α-methylsterols and 30 mg other sterols.

Orobanche lutea: sterols

The results reported in Table 1 were obtained using an unsaponifiable fraction since the free and esterified sterols were not separated in the present work. In the 4,4-dimethylsterol fraction, no trace of lanosterol or 24-methylenelanostenol could be detected, whereas cycloartenol and 24-methylenecycloartanol were present. In the 4α-methylsterol fraction, in addition to the expected sterols (cycloeucalenol, obtusifoliol, 24-methylenelophenol and 24-ethylidenelophenol), minor sterols (especially 24,28-dihydrosterols) were also identified. Their structures were tentatively assigned on the basis of GLC and GLC–MS data. The 4-desmethylsterol fraction consisted essentially of C_{29}-sterols (sitosterol, stigmasterol and Δ^7-stigmastenol) with a smaller amount of C_{28}-sterols (campesterol and Δ^7-campestenol). The presence of Δ^7-avenasterol and isofucosterol on the one hand, and episterol and 24-methylene cho-

Table 1. 4,4-Dimethyl sterols, 4α-methyl sterols and sterols from *Orobanche lutea*

4,4-Dimethylsterols	(%)	4α-Methylsterols	(%)	Sterols	(%)
Cycloartenol	60	Cycloeucalenol	12	Cholesterol	tr.
24-Methylene cycloartanol	40	24,28-Dihydrocyclocucalenol	3	Episterol	tr.
		Obtusifoliol	13	24-Methylene cholesterol	tr.
		24,28-Dihydroobtusifoliol	3	Δ^7-Campestenol	4
		24-Methylene lophenol	8	Campesterol	4
		24-Methyl lophenol	2	Δ^7-Avenasterol	3
		4α-Methyl Δ^8-campestenol	2	*Iso*fucosterol	3
		24-Ethylidene lophenol	41	Δ^7-Stigmastenol	26
		24-Ethyl lophenol	6	Sitosterol	40
		4α-Methyl Δ^8-stigmastenol	2	Stigmasterol	22
		Not identified	9	Stigmastanol	tr.

lesterol on the other is in agreement with a biogenetic scheme previously postulated [5].

Biosynthetic investigations. Table 2 shows the radioactivity incorporated into 4,4-dimethyl sterols, 4α-methyl sterols and 4-desmethyl sterols respectively after a 24 hr incubation of *O. lutea* shoots with acetate-[^{14}C]. All these substances were heavily labelled and the high specific activities reflect a strong sterol biosynthetic activity in *O. lutea* tissues. The specific activities were, as expected, higher for the postulated precursors than for the products.

Since radioactivity was detected in the lanosterol carrier (as the diepoxide–acetate, $1{\cdot}79 \times 10^4$ dpm of ^{14}C) and in the cycloartenol carrier (as the epoxide–acetate, $2{\cdot}05 \times 10^6$ dpm of ^{14}C), the two products were recrystallized to constant specific activity (Table 4).

Table 3 shows clearly that crystals from the epoxide of cycloartenol acetate retained all their radioactivity after the first crystallization and that the ^{3}H:^{14}C ratio remained constant during five crystallizations. In contrast carrier crystals of the diepoxide of lanosteryl acetate lost almost all their radioactivity after the first crystallization ($3{\cdot}2 \times 10^3$ dpm of ^{14}C), giving a negligible value after the third crystallization (10^3 dpm of ^{14}C). The ^{3}H:^{14}C ratio decreased continuously during the three crystallizations. We conclude that lanosterol is absent in *O. lutea*, and that sterol biosynthesis follows the normal cycloartenol pathway of higher plants.

Cuscuta species

Results obtained with *C. epithymum* and *C. europaea* are presented in Table 4. The 4-desmethylsterol composition of the two species was the same. Sitosterol and campesterol were the major 4-desmethylsterols in the two species but profound differences existed between the 4-desmethylsterol composition of *C. europaea* and the host plant *Nicotiana tabacum*. In particular much larger amounts of stigmasterol and cholesterol were present in the host than in the parasite. Thus, in *Nicotiana* the amounts of cholesterol, campesterol, sitosterol and stigmasterol were respectively 10, 20, 15 and 55%, whereas in *Cuscuta* the amounts were 1, 27, 65 and 7%.

After a 6 hr incubation of acetate-[^{14}C] with *C. europaea* radioactivity was detected in the sterols (3×10^5 dpm) and the triterpene fraction ($2{\cdot}3 \times 10^5$ dpm). Epoxidation of the triterpenes after addition of the appropriate carriers and separation of the components [6], showed that radioactivity was present in β-amyrin (10^5 dpm, identified also by MS) and in the monoepoxide of cycloartenyl acetate ($1{\cdot}5 \times 10^4$ dpm). A trace of radioactivity present in the diepoxide of lanosteryl acetate was lost after a second chromatography.

DISCUSSION

O. lutea and *C. europaea* were shown to be capable of synthesizing sterols and in all three plants

Table 2. Radioactivity incorporated into the acetates of sterols and their precursors after incubation of *O. lutea* shoots with acetate-[^{14}C] for 24 hr

	dpm
Cycloartenol	$2{\cdot}8 \times 10^6$
24-Methylenecycloartanol	$1{\cdot}2 \times 10^6$
Cycloeucalenol + obtusifoliol	$2{\cdot}8 \times 10^5$
24-Methylenelophenol	$6{\cdot}0 \times 10^5$
24-Ethylidenelophenol	$5{\cdot}6 \times 10^5$
Saturated, Δ^8 and Δ^7-4α-methyl sterols	$6{\cdot}8 \times 10^4$
Episterol + 24-methylenecholesterol	$5{\cdot}3 \times 10^4$
Δ^7-Avenasterol + isofucosterol	$2{\cdot}0 \times 10^5$
Δ^7 and Δ^5-sterols	$2{\cdot}6 \times 10^5$

Table 3. Recrystallizations of acetate of cycloartenol epoxide and acetate of lanosterol diepoxide

	Acetate of cycloartenol-epoxide ($^3H/^{14}C$)		Acetate of lanosterol diepoxide ($^3H/^{14}C$)	
	Crystals	Mother liquor	Crystals	Mother liquor
Before recrystallization	2·09		3·01	
(1) Recrystallization	2·11	2·13	1·46	2·20
(2) Recrystallization	2·09	2·14	1·07	1·33
(3) Recrystallization	2·09	2·10	0·58	0·82
(4) Recrystallization	2·13	2·12		
(5) Recrystallization	2·14	2·12		

Table 4. 4,4-Dimethylsterols, 4α-methylsterols and sterols of *Cuscuta epithymum* ssp *trifolii*

4,4-Dimethyl sterols	(%)	4α-Methyl sterols	(%)	Sterols	(%)
Cycloartenol	28	Cycloeucalenol	48	Cholesterol	2
24-Methylene cycloartanol	72	Obtusifoliol	18	Episterol	tr.
		24-Methylene lophenol	13	24-Methylene cholesterol	1
		24-Ethylidene lophenol	20	Campesterol	21
				Δ^7-Avenasterol	tr.
				*Iso*fucosterol	5
				Sitosterol	64
				Stigmasterol	8

examined cycloartenol derivatives were identified, whereas lanosterol was absent.

It has been pointed out that photosynthetic eucaryote phyla are characterized by a sterol biosynthetic pathway involving cycloartenol. By contrast non-photosynthetic eucaryote phyla are characterized by a pathway involving lanosterol [1]. This observation raises the following question: does the presence of a functional photosynthetic apparatus determine the sterol biosynthetic pathway at the level of 4,4-dimethylsterols? We have shown that green and etiolated *Euglena gracilis* do not display appreciable differences in their 4,4-dimethylsterols [2]. Results obtained in the present work and the observations of Nes *et al.* on pine endosperm [4] confirm the presence, in non-photosynthetic tissues of higher plants, of the typical plant pathway. Therefore the absence of functional chloroplasts in plants does not lead to modification in the nature of 4,4-dimethylsterols involved in sterol biosynthesis. Furthermore, results obtained with *Astasia longa* [3], a natural apoplastid mutant of *Euglena*, support the above conclusion since, even in the total absence of chloroplasts, cycloartenol derivatives retain their role.

On the one hand differences exist between photosynthetic eucaryotes and non-photosynthetic eucaryotes, at the level of 4,4-dimethylsterols involved in sterol biosynthesis. On the other hand the absence of chloroplasts does not modify the nature of the 4,4-dimethylsterols present in the eucaryotes belonging to a photosynthetic phylum. These two facts suggest the hypothesis that the chloroplast, even if it does not play a direct role in the orientation of the sterol biosynthesis pathway, has nevertheless been able to induce the cycloartenol pathway. This is in agreement with the hypothesis that photosynthetic eucaryotes existing today could have arisen from a fusion between photosynthetic procaryotes (blue–green algae) and non-photosynthetic eucaryotes [7]. Such examples of metabolic modifications following the integration of a foreign organism into a host cell do exist. For example infection of plant cells with *Agrobacterium tumefaciens* [8], infection of legumes with *Rhizobium* [8] and symbiosis between algae and fungi in lichens [7] lead to the production of new metabolites absent in each symbiont alone or modify preexisting metabolic pathways.

We believe that the integration of a procaryotic blue-green algae into the ancestral eucaryote either would have modified the preexisting metabolic pathway involving lanosterol, or would have promoted the synthesis of new products, for example cycloartenol, in an organism which was previously unable to synthesize this compound.

EXPERIMENTAL

TLC was carried out on Merck HF 254 plates (0·2 mm). The plates impregnated with $AgNO_3$ were immersed in a 10% soln of $AgNO_3$ in EtOH–H_2O (3:1), dried for 12 hr at room temp and activated for 30 min at 110°. After spraying with a 0·1% soln of berberin hydrochloride in EtOH, the products were observed under UV (340 nm).

GLC employed FID and glass columns (1·50 m × 3 mm) packed with either SE-30 (1%), OV-17 (1%) or XE-60 (1%) on Chromosorb WHP (100–120 mesh). The alcohols were analysed at 255° on OV-17 and SE-30, the acetates at 270° on OV-17 and SE-30, at 240° on XE-60. The retention indices were compared to those of reference compounds [10, 11]. The quantity of sterol was measured by comparing the peak areas with that of an internal reference of *n*-dotriacontane (average error about 5%).

GLC–MS was carried out on a LKB 9000 S mass spectrometer at an ionizing energy of 70 eV. The separation was carried out at 270° on a glass column (4 m × 5 mm) packed with Dexsil (1%).

Radioactivity was measured by liquid scintillation counting.

Plant material. Orobanche lutea Baumgartn. was gathered at the Goeftberg near Strasbourg in June 1973 and 1974, *Cuscuta epithymum ssp. trifolii* L. at St-Pierre-sur-Dives (Calvados) in September 1973 in a field of alfalfa. *Cuscuta europaea* L. seeds were sterilized by soaking in 20% calcium hypochlorite and after thorough washing were germinated under sterile conditions in Petri dishes at 24°. Germination commenced after 8–9 days. When the shoots were 1–2 cm long they were placed in contact with the stem of the intended host plant (*Nicotiana tabacum* var. Samsun) just under the surface of the soil. The host plants were kept in a greenhouse until the parasite had developed sufficiently for harvesting.

Incubation procedure. The cut stems of five *O. lutea* plants were placed in distilled water (4 ml) containing 1 mCi of sodium acetate-[2-^{14}C] (specific activity 52 mCi/mM, CEA Saclay) for 24 hr. *C europaea* was cut into segments 1–2 cm long and moistened with an aq. soln of 0·1 mCi of acetate-(2-^{14}C) for 6 hr.

Analytical procedures. The plant material was lyophilised, homogenized with Me_2CO(250 ml) refluxed for 1 hr and extracted 2× with 250 ml $CHCl_3$–MeOH (2:1) for 2 hr. After filtration the extracts were combined and evaporated to dryness under vac. The extract was then saponified for 1 hr under reflux with 150 ml of a 6% solution of KOH in MeOH with 250 mg pyrogallol. The non-saponifiable lipids were extracted and separated by TLC on silicagel (two migrations using CH_2Cl_2) into 4,4-dimethylsterols, 4α-methyl sterols and sterols. These three fractions were acetylated at room temp for 18 hr with a 20% soln of acetic anhydride-[^{3}H] in C_6H_6 (CEN, Mol, Belgium; sp. activity 12·5 mCi/mM for sterol precursors and 1 mCi/mM for sterols). The acetates were purified by TLC using C_6H_6–EtOAc (9:1) as developing solvent. The three acetate fractions were separated by TLC on silica gel impregnated with $AgNO_3$ by continuous development for 16 hr using cyclohexane–C_6H_6 (7:3). The steryl acetates were separated into four bands which were analysed by GLC–MS. *Band* 1: stigmastanyl acetate, *m/e* 458, 443, 398, 283, 290, 257, 230, 215. *Band* 2: cholesteryl acetate (traces); campesteryl acetate, *m/e* 382, 367, 255, 213; Δ^7-campestenyl acetate *m/e* 442, 427, 382, 367, 315, 273, 255, 213, sitosteryl acetate, *m/e* 396, 381, 255, 213; Δ^7-stigmastenyl acetate, *m/e* 456, 441, 396, 381, 315, 289, 273, 255, 229, 213; stigmasteryl acetate, *m/e* 394, 379, 282, 255, 213. *Band* 3: isofucosteryl acetate, *m/e* 454, 439, 394, 379, 313, 296, 271, 253, 213; Δ^7-avenasteryl acetate, *m/e* 454, 439, 394, 379, 356, 341, 327, 313, 296, 255, 253, 213. *Band* 4: 24-methylene cholesteryl acetate, *m/e* 440, 425, 380, 365, 356, 296, 255, 253; episteryl acetate, *m/e* 440, 425, 380, 365, 356, 313, 296, 273, 255, 253, 229, 213. The 4α-methyl steryl acetates were resolved into 4 bands. *Band* 1: the components of this mixture were tentatively identified by GLC and GLC–MS analysis. The positions of the double bond at the Δ^8 or Δ^7 position were deduced from the retention time on GLC and by the relative intensities of the fragmentations. 24,28-Dihydro cycloeucalenyl acetate, *m/e* 470, 455, 410, 395, 343, 302, 283, 175; 24,28-Dihydro obtusifoliyl acetate *m/e* 470, 455, 410, 395; 24-methyl lophenyl acetate, *m/e* 456, 441, 396, 381, 329, 327, 269, 243, 227; 24-ethyl lophenyl acetate, *m/e* 470, 455, 410, 395, 329, 269, 243, 227; 4α-methyl-Δ^8-campestenyl acetate, *m/e* 456, 441, 396, 381, 269, 243, 227; 4α-methyl-Δ^8-stigmastenyl acetate, *m/e* 470, 455, 410, 395, 269. *Band* 2: 24-ethylidene lophenyl acetate, *m/e* 468, 453, 408, 393, 370, 327, 310, 267, 227. *Band* 3: Cycloeucalenyl acetate, *m/e* 468, 453, 408, 393, 365, 353, 343, 325, 300, 283; obtusifoliyl acetate, *m/e* 468, 453, 393, 287, 227. *Band* 4: 24-methylene lophenyl acetate, *m/e* 454, 449, 394, 379, 370, 355, 341, 327, 287, 269, 267, 227, 207. The 4,4-dimethylsteryl acetates were resolved into two bands. *Band* 1: Cycloartenyl acetate; in the case of *C. epithymum* this fraction contained great quantities of unknown products and the MS of cycloartenyl acetate could not be recorded. *Band* 2: 24-methylenecycloartanyl acetate, *m/e* 482, 467, 439, 422, 407, 379, 355, 300.

In the case of the *O. lutea* and *C. europaea* incubations with acetate-[2-^{14}C] 0·5 mg each of lanostenyl acetate and cycloartenyl acetate was added as carrier before the TLC separation of the labelled 4,4-dimethylsteryl acetates on $AgNO_3$-Silica gel. After separation, 1 mg of lanosteryl and cycloartenyl acetates was added to Band 1, and the fraction was epoxidised with a saturated soln of *p*-nitroperbenzoic acid in Et_2O, and the epoxi-acetates separated by TLC with two migrations with cyclohexane-EtOAc (85:15) into the acetates of cycloartenyl monoepoxide and lanosteryl diepoxide [6]. After addition of 17 mg of carrier, the epoxy-acetates were recrystallized in MeOH.

Acknowledgements—We thank Prof. A. Gagnieu and Dr. J. Meyer who provided us with *C. europaea* seeds, and Dr. G. Coutière for *C. epithymum* plants. One of us (T.B.) was in receipt of a Royal Society European Fellowship.

REFERENCES

1. Goad, L. J. (1971) in *Natural substances formed biologically from mevalonic acid* (Goodwin, T. W., ed.). Academic Press, London.
2. Anding, C., Brandt, R. D. and Ourisson, G. (1971) *Eur. J. Biochem.* **24,** 259.
3. Rohmer, M. and Brandt, R. D. (1973) *Eur. J. Biochem.* **36,** 446.
4. Malhotra, H. C. and Nes, W. R. (1972) *J. Biol. Chem.* **247,** 6243.
5. Goad, L. J. (1972) *Progress in Phytochemistry*, Vol. 3, pp. 113–198. Interscience, London.
6. Ponsinet, G. and Ourisson, G. (1968) *Phytochemistry* **7,** 757.
7. Margulis, L. (1970) *Origin of eukaryotic cells.* Yale University Press. New Haven and London.
8. Tourneur, J. and Morel, G. (1971) *Physiologie Végétale* **9,** 527.
9. Dilworth, M. J. and Parker, C. A. (1969) *J. Theoret. Biol.* **25,** 208.
10. Ettre, L. S. (1964) *Anal. Chem.* 36, (8), 31A.
11. Patterson, G. W. (1971) *Anal. Chem.* **43,** 1165.