Oxidation of 3 *to* 2. The alcohol (3, 20 mg) in Me_2CO (3 ml) was treated with an excess of Jones reagent at 0° for 5 min. The product extracted with Et_2O was submitted to preparative TLC to give the α,β-unsaturated ketone (2, 15 mg) which was crystallized from EtOH as needles: $C_{20}H_{30}O$ (M^+ 286), mp 99–100°, $[\alpha]_D$ −146°.

Acetylation of 3 *to* 4. The diterpenoid (3, 10 mg) was treated with Ac_2O (0.5 ml) in dried pyridine (0.5 ml) at room temp for 15 hr. The product 4 (7 mg) $C_{22}H_{34}O_2$ (M^+ 330) had mp 115–116°, $[\alpha]_D$ −100°.

Rearrangement of 3 *to* 1. The unsaturated alcohol (3, 20 mg) in MeOH (4 ml) and ether (2 ml) was treated with conc NCl (0.8 ml) at room temp. for 3 hr. The reaction mixture was taken up into Et_2O, and the ethereal solution was washed, dried and concd to give a major product (1, 15 mg) which was submitted to preparative TLC and recrystallization: $C_{20}H_{32}O$ (M^+ 288), mp 148–149°, $[\alpha]_D$ −78°.

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IN VITRO CYCLIZATION OF SQUALENE 2,3-EPOXIDE TO α-AMYRIN BY MICROSOMES FROM BRAMBLE CELL SUSPENSION CULTURES

JOHN W. ELDER*, PIERRE BENVENISTE† and PAUL FONTENEAU†

*Department of Chemistry, Fairfield University, Fairfield, Conn. 06430, U.S.A.; †Institut de Botanique, Laboratoire de Biochimie Végétale, E.R.A. au C.N.R.S. n° 487, 28, rue Goethe, 67083 Strasbourg Cedex, France

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Key Word Index—*Rubus fruticosus*; Rosaceae; squalene 2,3-epoxide-α-amyrin cyclase.

Abstract—Squalene 2,3-epoxide incubated with microsomes from bramble cell suspension cultures is shown to be converted into α-amyrin.

INTRODUCTION

In 1955, Ruzicka and his coworkers proposed the biosynthesis of α- and β-amyrin, along with other triterpenes and steroids from squalene [1]. The *in vitro* incorporation of mevalonate and squalene 2,3-epoxide into β-amyrin has been demonstrated [2–5]. In the case of α- amyrin, *in vivo* experiments have shown incorporation of CO_2, acetate and mevalonate into α-amyrin or its derivatives [6–8]. Recently incorporation of mevalonate into α- and β-amyrin by chopped preparations of plant parts has been shown [9]. *In vitro* biosynthesis of α-amyrin has not been reported; however, Corey and Dean imply that less than 5% of the radioactivity found in impure β-amyrin from *in vitro* incorporation of squalene-epoxide might be due to α-amyrin but offer no proof [5, 10]. In many cases, α-amyrin seems to constitute a minor component of the pentacyclic triterpene mixture and this, added to the difficulty to separate the two amyrins, rendered very difficult the study of *in vitro* biosynthesis of α-amyrin. As we recently showed that α-amyrin constituted more than 70% of the pentacyclic triterpene fraction in bramble cells [11], we decided therefore to study the *in vitro* biosynthesis of this compound. We wish to report here an unambiguous incorporation of squalene epoxide into α-amyrin by microsomes from bramble cells suspension cultures.

RESULTS AND DISCUSSION

The 4,4-dimethyl steryl acetate fraction from bramble cell suspension cultures has been resolved by argentation chromatography into 24-methylene cycloartenyl acetate (R_f 0.20), cycloartenyl acetate (R_f 0.45) and pentacyclic triterpene acetates (R_f 0.80). GC–MS analysis showed that this latter fraction contained β-amyrin

Table 1. GLC and MS data of amyrin acetates

Triterpene acetate	GLC data RR_t (cholesterol = 1.00)		MS data (in GC–MS, 70 eV), m/e and relative intensities (%)					
	SE-30 (240°)	OV-17 (270°)	M^+	a	b	c	d	e
β-amyrin acetate	2.23	2.10	468 (3)	453 (1)	408 (1)	393 (2)	218 (100)	203 (34)
α-amyrin acetate	2.45	2.38	468 (3)	453 (1)	408 (1)	393 (2)	218 (100)	203 (16)

acetate (30%) and α-amyrin acetate (70%) (Table 1). These two products have been separated on a large scale by selective epoxidation. These conditions result in the expoxidation of more than 99% of the β-amyrin acetate while leaving more than 97% of the α-amyrin acetate unreacted.

Mevalonate-5-^{14}C has been incubated in the presence of a post mitochondrial supernatant fortified with ATP and NADPH. Radioactivity has been obtained in squalene (3.12×10^6 dpm) and in 4,4-dimethyl sterols (0.44×10^6 dpm). After acetylation of the 4,4-dimethyl sterol fraction, argentation chromatography of the acetates, and epoxidation of the pentacyclic triterpene acetates, the unreacted α-amyrin acetate was separated from β-amyrin acetate epoxide, unlabelled carriers were added and the α-amyrin acetate and the β-amyrin acetate epoxide have been crystallized to specific constant radioactivity (Table 2). As shown here, total radioactivity associated with α-amyrin (313600 dpm) appeared to be much higher than that associated with β-amyrin (46000 dpm).

Squalene 2,3-epoxide was incubated in the presence of microsomes as described previously [12]. Using the same procedure as before, the separated α-amyrin acetate and β-amyrin acetate epoxide were crystallized to specific constant radioactivity (Table 3). A specific constant radioactivity was obtained after the second crystallization in the case of α-amyrin acetate which constitutes direct evidence for the *in vitro* formation of α-amyrin from squalene epoxide. Such a result is of importance; we think that our system will be useful for investigating further aspects of the biosynthesis of α-amyrin.

EXPERIMENTAL

TLC was carried out on Merck HF 254 plates (0.2 mm). After spraying with 0.1% berberine–HCl in EtOH, the products were observed under UV (340 nm). GLC employed a GC fitted with two FID and two glass columns (1.50 m × 3 mm) packed with either 1% SE30 or 1% OV-17 and N_2 at 30 ml/min. GC–MS was carried out on a LKB-9000 S mass spectrometer at an ionizing energy of 70 eV. Radioactivity on the TLC plates was detected by a thin layer scanner. Radioactivity was assayed by liquid scintillation counting.

Radiochemicals. Mevalonolactone-(5-^{14}C) (11 μCi/μmol) was supplied by the Commissariat á l'Energie Atomique (Gif-sur-Yvette, France). Squalene-(11,12-^{3}H) was prepared as described previously [13]. Squalene-(11, 12-^{3}H)-2,3-epoxide was synthesized by the method of van Tamelen and Curphey [14].

Preparation of subcellular fractions. Bramble cells (*Rubus fruticosus*) were grown under continuous white light at 25° on

Table 2. Crystallization to constant specific radioactivity of α-amyrin acetate and the epoxide of β-amyrin acetate obtained after incubation of mevalonate-(5-^{14}C)

	α-Amyrin acetate		Epoxide of β-amyrin acetate	
Amount of unlabelled product added (mg)	40		40	
Radioactivity before crystallization (dpm/mg)	7840 ± 400		1150 ± 40	
	Crystals	m.l.	Crystals	m.l.
1st crystallization (dpm/mg)	9250 ± 400	9100 ± 300	620 ± 15	4850 ± 40
2nd crystallization	9200 ± 400	8800 ± 500	440 ± 15	1100 ± 20
3rd crystallization	9100 ± 400	9600 ± 500	340 ± 15	950 ± 20
4th crystallization	9400 ± 400	9350 ± 500	325 ± 20	450 ± 20
5th crystallization	—	—	325 ± 20	345 ± 20
6th crystallization	—	—	330 ± 25	340 ± 25

Table 3. Crystallization to constant specific radioactivity of α-amyrin acetate and the epoxide of β-amyrin acetate obtained after incubation of squalene-(11, 12-^{3}H)-2,3-epoxide

	α-Amyrin acetate		Epoxide of β-amyrin acetate	
Amount of unlabelled product added (mg)	30		30	
Radioactivity before crystallization (dpm/mg)	1760 ± 60		230 ± 20	
	Crystals	m.l.	Crystals	m.l.
1st crystallization (dpm/mg)	1450 ± 60	1450 ± 60	190 ± 20	280 ± 20
2nd crystallization	1150 ± 60	1200 ± 60	200 ± 20	200 ± 20
3rd crystallization	1300 ± 60	1100 ± 60	190 ± 20	190 ± 20
4th crystallization	1150 ± 60	1050 ± 80	185 ± 20	200 ± 20

a synthetic medium having the following composition: mineral sol of Heller [15] (100 ml), thiamine (1 mg), glucose (50 g), and 2 × dist. H_2O (900 ml). Cells were harvested during the exponentially growing phase. Cells (100 g) were ground in a mortar at 0° with 1 vol. of medium containing: 0.1 M Tris–HCl, 1 mM EDTA, 10 mM mercaptoethanol, 0.5 M sucrose, final pH 7.5. Homogenate was squeezed through 2 layers of cheese-cloth and centrifuged at 6000 *g* for 10 min. Supernatant was centrifuged at 105000 *g* for 60 min. Soluble supernatant was removed and microsomal pellets suspended in 5 ml medium containing: 0.1 M Pi buffer, 2.5 mM mercaptoethanol, 4 mM $MgCl_2$, final pH 7.5 and dispersed in a Potter–Elvehjem homogenizer. Because of progressive acidification, the pH was adjusted to 7.5 with a 1 M Tris soln.

Enzymatic assays. Incubation of squalene epoxide. Dispersed microsomal pellets (4.5 ml; 4 mg protein/ml) were incubated with squalene-(11, 12-^{3}H)-2,3-epoxide ($12 . 10^6$ dpm; 250 μM) dissolved in 0.5 ml 0.1 M Pi buffer containing 1% Tween-80 for 4 hr at 31°. The reaction was terminated by the addition of 20% KOH in EtOH (1 vol.) In one expt a duplicate consisting of particles boiled for 30 min was used. Protein was quantitated by the procedure of Lowry *et al.* [16].

Incubation of mevalonate. A 6000 *g* supernatant (20 ml) was incubated in the presence of ATP (3.6 mM), NADPH (1.2 mM): $MgCl_2$ (4 mM) and mevalonate. 5-^{14}C (10 μCi) for 30 min at 30°C. The reaction was terminated by the addition of 20% KOH in EtOH (1 vol.).

Analytical procedure. The incubation mixture was extracted 5 × with petrol (1 vol.). Combined extracts were dried, evaporated and subjected to Si gel TLC (CH_2Cl_2, two migrations). The band (R_f 0.35) containing 4,4-dimethyl sterols was eluted and acetylated. Acetylated materials was chromatographed on $AgNO_3$–Si gel plates using C_6H_6–cyclohexane (3:7; continuous development for 16 hr), 3 bands containing respectively 24-methylene cycloartanyl acetate (R_f 0.20), cycloartenyl acetate (R_f 0.45) and the amyrin acetates (R_f 0.80) were obtained, and material in the band containing the amyrin acetates was treated with a saturated soln of *p*-nitroperbenzoic acid in Et_2O (1 ml) over a period of 2 days at room temp. [16]. The Et_2O soln was washed with $Na_2S_2O_3$ and Na_2CO_3 solns, dried, concd and subjected to TLC (CH_2Cl_2, 1 migration). The band containing unreacted acetate (R_f 0.75) and epoxidized acetate (R_f 0.50) were eluted and α-amyrin acetate and β-amyrin acetate epoxide respectively added as carriers. The 2 samples were recrystallized 4 × from MeOH.

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INHALTSSTOFFE DER GATTUNG *POLYMNIA*

FERDINAND BOHLMANN und CHRISTA ZDERO

Institut für Organische Chemie der Technischen Universität, 1000 Berlin 12, Straße des 17. Juni 135, W. Germany

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Aus der Gattung *Polymnia*, die zur zweiten Untergruppe des Subtribus *Melampodiinae* gehört [1], sind bereits mehrere Arten untersucht. Man findet hier neben dem weitverbreiteten Pentainen **1** [2] vor allem verschiedene Germacrolide [3, 4] sowie einmal ein Eudesmanolide [4].

Die Wurzeln von *P. fructicosa* enthalten ebenfalls **1** sowie in relativ hoher Konzentration Kaurensäure [2] und die Derivate **3** und **4** [5]. Die oberirdischen Teile lieferten die Ester **5–8** sowie die Flavanone Naringenin **9** und Sakuranetin **10**. **6** und **7** sind bisher nicht als Naturstoffe isoliert worden, ihre Konstitutionen folgen eindeutig aus den spektroskopischen Daten.

Die Wurzeln von *P. pyramidalis* enthalten ebenfalls **1–4**, während die oberirdischen Teile **2** und **6** ergeben. In beiden Arten konnten keine Sesquiterpenlactone