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THE BIOSYNTHESIS, METABOLISM AND TRANSLOCATION OF β-AMYRIN IN *SORGHUM BICOLOR*

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Key Word Index—*Sorghum bicolor*; Graminae; polycyclic isopentenoids; α-amyrin; δ-amyrin; sorghumol; β-amyrone; sterols.

Abstract—The biosynthesis of [^{14}C]β-amyrin and three other labelled 3β-hydroxypentacyclic triterpenes from [2-^{14}C]acetate in leaves of *Sorghum bicolor* was demonstrated. Evidence for the metabolism of [^{14}C]β-amyrin to the corresponding C-3 ketone (β-amyrone) and for the transport of [^{14}C]β-amyrin in leaves was also obtained.

INTRODUCTION

Much is known about the biosynthesis of β-amyrin in plants [1–7]. However, how the plant utilizes it metabolically and functionally is not fully understood. There is evidence that β-amyrin can be converted to C-3 methyl-ethers [8], cinnamates [9–12] and other esters [9–12], or oxidized at various positions in the ring system, e.g. to produce erythrodiol and oleanolic acid [13]. β-Amyrone, a C-3 oxopentacyclic triterpene (C-3 ketone), and seco-pentacyclic triterpenes, believed to be derived either photochemically or photomimetically from β-amyrone, have been detected in sediments and petroleum [14]. Recently, the co-occurrence of 3β-hydroxy-, C-3 oxo- and seco-pentacyclic triterpenes in the surface wax of several *Hoya* species [15] has indicated the possibility that 3β-hydroxypentacyclic triterpenes, such as β-amyrin, are enzymatically converted, via their corresponding C-3 ketones, to seco-derivatives, the latter presumably having a novel role in the plants interaction with its biotic environment [15–19]. Additionally, there is some speculation for a role of 3β-hydroxypentacyclic triterpenes in membrane systems [20]. We have previously determined the polycyclic isopentenoid (sterol [21] and pentacyclic triterpene [22, 23]) composition of the leaf tissue and surface wax from flowering *Sorghum bicolor* (Moench) L. and here report the biosynthesis of [^{14}C]β-amyrin from [2-^{14}C]acetate, the metabolism of [^{14}C]β-amyrin to [^{14}C]β-amyrone and the translocation of [^{14}C]β-amyrin in the leaf. As an aid in the interpretation of the results for β-amyrin, similar information was obtained for related pentacycles, sterols and long chain fatty alcohols.

RESULTS AND DISCUSSION

β-Amyrin and three other 3β-hydroxypentacyclic triterpenes were actively biosynthesized from [2-^{14}C]acetate fed for seven days to mature leaves of *Sorghum bicolor* (Moench) L. cv IS 809; the specific activities of the pentacyclic triterpene pool (free alcohols and esters) and the isolated 3β-hydroxypentacycles are given in Tables 1 and 2, respectively. Preliminary investigations had indicated that the total pentacyclic triterpene, sterol and long chain fatty alcohol (LCFA) pools of the leaf (plus wax) in developmental stages equivalent to that of the leaf at the time of the [2-^{14}C]acetate treatment and at the time of harvest (seven days later) did not vary to a measurable extent. The purified pentacyclic triterpene fraction, obtained from the nonsaponifiable lipid fraction (NLF), and thus representing the sum of the free alcohol and ester pools [23], had a specific activity higher than that of other structural components, i.e. sterols and LCFA, isolated from the treated leaf (Table 1). A portion of the pentacyclic triterpene fraction was examined by GLC, acetylated and reexamined by GLC–MS (Fig. 1). The 3β-hydroxypentacyclic triterpenes listed in Table 2 were tentatively identified by a comparison of their mass spectra to standards obtained as described in the accompanying paper [23]. In addition, a trace amount of 24-methylenecycloartanol (Peak D, Fig. 1) was identified through a comparison of its chromatographic properties (GLC) and its mass spectra to that of a standard and to those values reported for the acetylated derivative in the literature [24]. 24-Methylenecycloartanol was probably present in the three varieties examined in this and the previous report [23] at trace levels making its detection highly variable. The pentacyclic triterpene fraction was further resolved into four fractions containing (GLC–MS, ^{1}H NMR [23]) β-amyrin, α-amyrin, δ-amyrin and sorghumol by RP-HPLC [23] in systems I and II (Table 2). Each of these fractions contained significant ^{14}C label from the feed (Table 2). No mass or radioactivity were detected at the α_cs of fernenol, isoarborinol or simiarenol (migrated hopanoids detected in other *S. bicolor* varieties, cf. [23]) further proving that mature leaves of IS 809 do not biosynthesize or contain these migrated hopanoids.

The specific activities of the purified 3β-hydroxypentacyclic triterpenes are compared to those of sterols isolated from a similar [2-^{14}C]acetate feed to IS 809 [21] in Table 2. From the data it is evident that mature IS 809 actively biosynthesizes β-amyrin and the

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Table 1. Specific activities of the lipid fractions from the [2-^{14}C]acetate feed to *S. bicolor* cv IS 809

Fraction	Radioactivity dpm ($\times 10^7$)	Weight mg	Specific activity dpm/mg ($\times 10^5$)
TLE*	2.40	87.10	2.75
NLF†	1.15	36.20	3.17
Sterols‡	0.046	2.40	1.92
LCFA§	0.018	0.645	2.79
Pentacyclic triterpenes‖	0.30	2.80	10.71

*Total lipid extract obtained by acetone extraction of the leaf material.

†Non-saponifiable lipid fraction obtained by saponifying the TLE in 10% methanolic KOH followed by work up in the usual manner.

‡Sterol mixture (cholesterol, campesterol, sitosterol and stigmasterol [21, 23] obtained from the alumina CC (70% diethyl ether–30% hexane, fraction). Mass was determined gravimetrically.

§Long chain fatty alcohol mixture (C_{22}, C_{24}, C_{26}, C_{28}, C_{30} chain lengths) obtained from Sephadex LH-20 column developed isocratically with 5% methanol in hexane, elution vol. 240–300 ml.

‖3β-Hydroxypentacyclic triterpene mixture (Fig. 1) obtained from Sephadex LH-20 column, elution vol. 340–470 ml.

Table 2. Specific activities of the polycyclic triterpenes isolated from a 7 day feed of [2-^{14}C]acetate to *S. bicolor* cv IS 809 leaves

Polycyclic triterpene	Specific activity (dpm/μg)
3β-Hydroxypentacyclic triterpenes	
β-amyrin	2007
δ-amyrin	1827
α-amyrin	3446
sorghumol	3424
Sterols*	
cholesterol	1036
campesterol	389
stigmasterol	39
sitosterol	416

*The specific activities of the sterols were determined in a previous feed of [2-^{14}C]acetate to leaves of *S. bicolor* cv IS 809 [21].

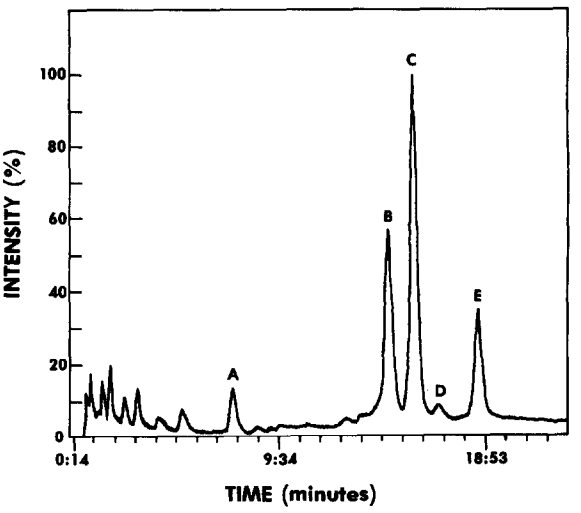

Fig. 1. Total ion current profile from the GLC-MS analysis of the acetylated 3β-hydroxypentacyclic triterpene fraction from *Sorghum bicolor* cv IS 809 leaves treated with [2-^{14}C]acetate. A portion of the 3β-hydroxypentacyclic triterpene fraction was acetylated and applied to a 3% OV-17 column (Gas chrom Q, 100/120 mesh, 2 m × 1 mm i.d.). The column was held at 245° (10 min), then programmed to 290° at 3° min^{-1}. MS conditions: electron current 70 eV, electron emission current 200 μA and source temperature 160°. Mass scanning was performed, immediately after injection, from 20 to 600 amu in 5.52 sec. A, cholesterol standard co-injected; B, β-amyrin; C, δ-amyrin and α-amyrin; D, 24-methylenecycloartanol (this peak was masked by other 3β-hydroxypentacycles in two other varieties of *S. bicolor* [23]); E, sorghumol.

three other pentacycles, the specific activity of each was of the same order of magnitude as cholesterol but at least an order of magnitude greater than the 24-alkylsterols which are probably the membrane structural sterols of sorghum [21]. The absence of significant radioactivity and mass associated with cycloartenol (identified in *S. bicolor* cv. G 499 GBR [23]) and significant radioactivity with 24-

methylenecycloartanol is further evidence of their dynamic turnover to sterols.

The high specific activity of β-amyrin, and each of the isolated 3β-hydroxpentacyclic triterpenes, relative to sterols (Table 2) was unexpected at this stage of development of the plants because of the presumed shift from pentacyclic triterpene to sterol synthesis during seed germination and seedling development (cf. [1]). This anomaly may be explained in part by the following scenarios: (1) Differences in the regulation of [^{14}C]squalene oxide cyclization to the 3β-hydroxypentacyclic triterpenes, derived from [2-^{14}C]acetate, versus sterols at this stage in the plants development may have resulted from a shift to the synthesis of pentacycles at the expense of the sterols; (2) Extensive metabolism of the 3β-hydroxypentacycles to oxidized products may have occurred at this developmental stage. For instance, β-amyrin and the other pentacycles may be metabolized, at different rates, through oxidation at C-3, C-24 or C-28 to give products such as secopentacyclic triterpenes, erythrodiol, oleanolic acid, medicagenic acid and sapogenols [13]. Alternatively, the sterols, especially the 24-alkylsterols (representing the bulk of the sterol pool) may be end-products without necessarily being further oxidized at this developmental stage. On the assumption that at the time of the [2-^{14}C]acetate feed, greater biosynthesis of β-amyrin (pentacyclic triterpenes) relative to the sterols took place (scenario 1) without, presumably, having undergone subsequent significant metabolism, then these compounds may have a specialized role in which intermembrane or intercellular transport/translocation away from the site of synthesis is involved. This latter point was amenable to experimental examination.

The metabolism and translocation of β-amyrin were investigated simultaneously by applying [^{14}C]β-amyrin (497 170 dpm; 2 mCi/mMol) to the tip of a mature leaf, incubating the plant for 72 hr and sectioning the leaf into a 'treated tip' and five leaf sections, each 5 cm in length, beginning 4 cm from the treatment spot and progressing toward the shoot. The radioactivity detected in the total lipid extracts (TLEs) of the treated tip and the five leaf sections and the specific activity, defined as the radioactivity detected in each TLE divided by the dry weight (mg), of each are given in Table 3.

Evidence was obtained for the metabolism of [^{14}C]β-amyrin to β-amyrone at the point of treatment. Most of the ^{14}C label in the TLE (212 000 dpm, 43% of the feed) from the treated tip was associated with β-amyrin (85% of the TLE radioactivity) through successive TLC as the free alcohol and acetate in systems I and II respectively and through HPLC–RC in systems I and II. No evidence was obtained for the metabolism of β-amyrin to other 3β-hydroxypentacyclic triterpenes endogenous to IS 809. Twelve percent of the label from the treated leaf tip TLE was recovered from the broad pentacyclic triterpene C-3 ketone + ester band following TLC (system I). The pentacyclic triterpene ketones and esters were separated in TLC system II and 47% of the label applied to the TLC plate was recovered from the ester region. The C-3 ketone fraction, containing 50 ng [^{14}C]β-amyrone (6700 dpm) with 533 μg carrier added, was chromatographed again in TLC system II and the recovered β-amyrone (2555 dpm, 530 μg) recrystallized to constant specific activity following the addition of 9.6 mg authentic β-amyrone (Table 4). Further confirmation of the radiochemical purity of the β-amyrone was obtained by reducing the C-3 ketone with $LiAlH_4$ to the free alcohol (an epimeric mixture), purifying the 3β-from the 3α-epimer by TLC (system I) and determining the specific activity of the resultant β-amyrin sample as 177 dpm/mg. From the final specific activity, it can be calculated that at least 0.4% of the net feed (497 170 dpm, 46.8 μg) was metabolized to [^{14}C]β-amyrone, i.e. final specific activity 177 dpm/mg × 10.1 mg carrier = 1790 dpm or *ca* 0.4% of the net feed. In a similar feed of [2-^{3}H]lanosterol, an isomer of the sterol precursor cycloartenol, to a sorghum leaf only 20% of the radioactivity in the leaf TLE was associated with 4-desmethylsterols; 80% was recovered unmetabolized (the metabolism of [2-^{3}H]lanosterol is the subject of another communication). If it is assumed that only the [2-^{3}H]lanosterol that enters the leaf cells is metabolized, and that all that enters is metabolized, then we may estimate that only 20% of the applied [^{14}C]β-amyrin, which probably has solubility properties similar to lanosterol in this system (barring selective uptake), was

Table 3. Translocation of [^{14}C]β-amyrin and [4-^{14}C]cholesterol in leaves of *S. bicolor* cv IS 809 after 72 hr

Treatment		Leaf position (cm)*					
		Origin	6.5	11.5	16.5	21.5	26.5
[^{14}C]β-amyrin (497 170 dpm)	dpm†	206 367	342	159	357	325	205
	mg dry wt‡	12.0	12.0	12.5	36.6	42.5	56.5
	sp. act.	17 197	28	13	7	8	4
[4-^{14}C]cholesterol (1 070 000 dpm)	dpm	770 895	3108	127	105	85	0
	mg dry wt	47.2	136.0	59.8	100.5	76.3	100.8
	sp. act.	16 343	22	2	1	1	0

*Leaf positions are the linear distance from the application spot (origin) to the center of each 5 cm section.

†Radioactivities were determined for aliquots of the TLE from each leaf section; each count was significantly above background (at least 10 × background).

‡Dry weight (mg) is the dry weight of the treated leaf tip (origin) and the five leaf sections (5 cm length).

Table 4. Recrystallization of [^{14}C] β-amyrone isolated from the [^{14}C-] β-amyrin feed to mature leaves of *S. bicolor**

	dpm/mg
Initial*	252
Acetone	176
Hexane	180
Ethanol–water	177

*Initial sample contained 2555 dpm, 50 ng β-amyrone and 10.1 mg carrier β-amyrone.

available for metabolism. Thus it can be calculated that at least 2% of the available [^{14}C]β-amyrin is metabolized to [^{14}C]β-amyrone. After accounting for losses during extraction and chromatographic procedures this value may be as high as 5%. Interestingly β-amyrone (estimated by GLC) represents *ca* 4% of the total β-amyrone + β-amyrin pool endogenous to the leaf at this developmental stage; β-amyrone was detected at the level of 4.0 μg/g dry wt and β-amyrin at 104.3 μg/dry wt.

The vectorial translocation (toward the shoot) of [^{14}C]β-amyrin in the leaf is demonstrated through the detection of ^{14}C label from the feed in each of the leaf sections examined after 72 hr (Table 3). Data from a similar feed of [4-^{14}C]cholesterol (1.07×10^6 dpm, 20 mCi/mMol) to a leaf are given as a control and indicates that cholesterol is also vectorially transported in sorghum leaves. In 72 hr, label from the [^{14}C]β-amyrin feed was detected as far as 26.5 cm from the origin. The TLEs from leaf sections, excluding the treated tip, were combined and fractionated in TLC system I. Seventy-nine percent of the radioactivity in the combined TLE co-chromatographed with β-amyrin; 9.5% remained at the origin with hydrolyzable polar derivatives and 12.5% migrated to the solvent front with esterified pentacyclic triterpenes. No radioactivity was detected at the R_f corresponding to β-amyrone. The radioactivity at the R_f of β-amyrin was associated solely with this compound as determined by HPLC–RC in system I. This data seemingly indicates that β-amyrin and cholesterol [25] are vectorially translocated mainly as the free alcohol in mature leaves perhaps, as Nicholas and co-workers suggest for these two compounds in *Pelargonium hortorum* [26], bound to some carrier protein.

EXPERIMENTAL

Detailed conditions for reversed phase–HPLC (RP–HPLC), GLC, GLC-MS and ^{1}H NMR (200 m Hz) were presented in the previous publication [23]. The eluents for RP–HPLC (C18 column—Ultrasphere-ODS, 5 μ dp, 4.6 mm i.d. × 250 mm) were 96% (system I) or 90% Aq MeOH (system II) at a flow rate of 1.6 ml/min. Two systems were employed for TLC; system I: silica gel G (250 μm × 20 cm × 20 cm plates) eluted with C_6H_6–Et_2O (9:1) (23); system II: silica gel G eluted with C_6H_{14}–C_6H_6 (8:2). After development, radiolabelled compounds were located on the plate by radioscanning.

Seeds from *S. bicolor* (Moench) L. cv IS 809 were grown for approximately 3 months as described [23]. The plants were 36 cm tall with inflorescences just emerging.

[2-^{14}C]*Acetate feed to leaves of* IS 809. [2-^{14}C]Acetate (250 μCi, as the sodium salt [58.9 mCi/mmol] purchased from Amersham International Ltd.) was dissolved in a solution of 0.1% silicon oil (L-77) and 0.1% D,L-α-tocopherol in EtOH. The soln was applied along a 3 cm strip of the midrib and onto the ligule of four leaves from one shoot. After 7 days, the treated leaf was sectioned from the plant and dried in an Abderhalden apparatus for 24 hr. The dried tissue was crushed to a powder and extracted with refluxed Me_2CO for 18 hr to obtain a total lipid extract (TLE). The TLE (2.40×10^7 dpm/87.10 mg) was saponified and a non-saponifiable lipid fraction (NLF) (1.15×10^7, 36.20 mg) obtained [23]. The NLF was chromatographed on 3% deactivated Al_2O_3 [23] to separate the 3β-hydroxypentacyclic triterpene/LCFA mixture (4.60×10^6 dpm/8.7 mg) from the sterols (4.60×10^5 dpm/2.40 mg). The former fraction, after TLC in system I, was separated into a pure LCFA (1.80×10^5 dpm/0.645 mg) and a pure pentacyclic triterpene pool (3.00×10^6 dpm/2.80 mg) by chromatography on Sephadex LH-20 (23). The pentacyclic triterpene fraction was resolved into 3 fractions containing β-amyrin/δ-amyrin (as a mixture (α_c 1.15)), α-amyrin (α_c 1.28) and sorghumol (α_c 1.92) by RP–HPLC (system I). β-Amyrin and δ-amyrin were separated through further chromatography of the mixture in RP–HPLC system II, β-amyrin (α_c 1.05) and δ-amyrin (α_c 1.09), by repeated injections onto the column and collecting the leading and tailing edge. Chemical purity of each sample was confirmed by GLC on 3 columns (3% SE-30, 3% OV-17, 1% SP-1000, 235°) [23]. The identities of each isolated 3β-hydroxypentacyclic triterpene from the feed was determined by a comparison of their mass spectra (GLC-MS) and ^{1}H NMR (200 m Hz) spectra to those of standards isolated from *S. bicolor* as previously described [23].

Metabolism and translocation of [^{14}C]β-*amyrin.* [^{14}C]β-Amyrin (2 mCi/mmol) was prepared biosynthetically from feeds of [2-^{14}C]MVA to germinating *Pisum sativum* L. seedlings [19]. The labelled compound (0.25 μCi; 557 600 dpm) was dissolved in 100 μl of a 0.1% silicon oil and 0.1% D,L-α-tocopherol in EtOH soln and applied to a spot 8 cm from the tip of a mature leaf; the net feed (applied minus amount in substrate vial) was 497 170 dpm. After 72 hr, the leaf was cut into 5 sections, each 5 cm in length, beginning 4 cm from the treatment spot. The treated tip and 5 leaf sections were dried, crushed individually to fine powders and each extracted with refluxing Me_2CO to obtain their respective TLE.

[^{14}C]β-*Amyrin metabolism.* The TLE from the treated tip (212 000 dpm, 6.37 mg) was separated into 3 radioactive fractions by TLC in system I: hydrolyzable polar derivative fraction (R_f 0.0–0.1; 6153 dpm); free 3β-hydroxypentacyclic triterpenes (R_f 0.5; 180 444 dpm); and C-3 ketones + esters (R_f 0.8–1.0; 25 000 dpm) [23]. The free 3β-hydroxypentacyclic triterpene fraction was acetylated (pyridine–Ac_2O, 2:1; room temp; 24 hr), the solvents removed and the sample separated into four principle fractions by TLC system II: free 3β-hydroxypentacyclic triterpenes (R_f 0.07), C-3 ketones (R_f 0.25), C-3 acetates (R_f 0.43) and C-3 esters (R_f 0.53). Radioactivity was detected only in the pentacyclic triterpene C-3 acetate zone (148 884 dpm). The radioactive sample was deacetylated and 1/3 of the sample (with 20 μg each of β-amyrin, α-amyrin and sorghumol added) was injected into the HPLC (system I). The radioactivity in each of 60 one-minute fractions was determined; radioactivity was found to be associated only with β-amyrin (α_c 1.15).

The original C-3 ketone + ester sample (25 000 dpm) was also acetylated and then fractionated in TLC system II; radioactivity was detected at the origin (942 dpm), at the R_f of the C-3 ketones (6700 dpm) and at the R_f of the C-3 esters (11 777 dpm). The C-3

ketone sample (containing 50 ng β-amyrone GLC), with 533 μg added carrier β-amyrone prepared by oxidation (pyridinium chlorochromate in CH_2Cl_2) of an authentic sample of β-amyrin, was chromatographed again in the same TLC system. Additional carrier β-amyrone (9.6 mg) was added to the recovered β-amyrone sample (2555 dpm 530 μg) and the sample recrystallized to constant specific radioactivity (Table 3). The [^{14}C]β-amyrone (2.4 mg) was then reduced with $LiAlH_4$ (1 mg) to give the C-3 alochol (an epimeric mixture, 2.3 mg) which was chromatographed in TLC system I. The recovered [^{14}C]β-amyrin was then quantitated by GLC and the remainder of the sample radiocounted by liquid scintillation spectroscopy.

[^{14}C]*β-Amyrin translocation.* Aliquots of the TLEs from each of the five leaf sections were radiocounted by liquid scintillation spectroscopy. The TLEs were then combined, the sample (1288 dpm) separated by TLC system I into four fractions and the radioactivity in each fraction determined by liquid scintillation spectroscopy: hydrolyzable polar derivatives (123 dpm), free 3β-hydroxypentacyclic triterpenes (1015 dpm), C-3 ketones (0 dpm) and esters (161 dpm). The free 3β-hydroxypentacyclic triterpene fraction was then chromatographed on a reverse-phase TLC plate (KC18, Whatman) developed with MeOH–H_2O (95:5). The recovered free 3β-hydroxypentacyclic triterpene sample (985 dpm) (with 20 μg each of β-amyrin, α-amyrin and sorghumol) was injected into the HPLC (system I); radioactivity was found to be associated only with β-amyrin.

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