The Biosynthesis of β -Amyrin

MECHANISM OF SQUALENE CYCLIZATION

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1. β -Amyrin synthesized by pea seedlings in the presence of (3*RS*)-[2- ^{14}C , (4*R*)-4- $^3\text{H}_1$]mevalonic acid (for nomenclature see Cahn, Ingold & Prelog, 1956) was subjected to a series of degradations to locate the positions within the molecule of the incorporated tritium. 2. The location of five of the six labelled hydrogen atoms at C-3, C-9, C-18 and C-19 (two) confirms that the mechanism of cyclization of squalene expected from the biogenetic isoprene rule is functioning *in vivo*.

The formation of squalene involves the loss of a hydrogen atom from C-4 of each molecule of the precursor MVA.† By using the doubly labelled species [2- ^{14}C , (4*R*)-4- $^3\text{H}_1$]MVA and [2- ^{14}C , (4*S*)-4- $^3\text{H}_1$]MVA, Cornforth, Cornforth, Donninger & Popják (1966) have shown that this loss of hydrogen is stereospecific, the *pro-S*-hydrogen atom from C-4 of MVA being lost and the *pro-R*-hydrogen atom retained in each case. [The term '*pro-S*' is used according to the nomenclature of Cahn, Ingold & Prelog (1956), as extended by Hanson (1966) to naming the paired ligands *gg*, at a tetrahedral atom X_{ggij}.] Thus squalene biosynthesized from [2- ^{14}C , (4*R*)-4- $^3\text{H}_1$]MVA has a $^3\text{H}/^{14}\text{C}$ atomic ratio 6:6. It has also been shown (Rees, Mercer & Goodwin, 1966) that, when pea seedlings are incubated with [2- ^{14}C , (4*R*)-4- $^3\text{H}_1$]MVA, the $^3\text{H}/^{14}\text{C}$ ratios in squalene and the pentacyclic triterpene β -amyrin (I) isolated are the same as in the starting material. Thus no labelled hydrogen atoms are lost in the formation of β -amyrin from [2- ^{14}C , (4*R*)-4- $^3\text{H}_1$]MVA, a result consistent with the mechanism postulated by Eschenmoser, Ruzicka, Jeger & Arigoni (1955) for the cyclization of squalene to form β -amyrin and other triterpenes.

The present paper describes the results of degradative experiments designed to establish the location of the labelled hydrogen atoms in β -amyrin biosynthesized from [2- ^{14}C , (4*R*)-4- $^3\text{H}_1$]MVA.

EXPERIMENTAL

Radioactive substrates. (3*RS*)-[2- ^{14}C , (4*R*)-4- $^3\text{H}_1$]MVA (0.810 μmole containing 0.724 μC of $^{14}\text{C}/\mu\text{mole}$ and 7.70 μC

of $^3\text{H}/\mu\text{mole}$) was a kind gift from Professor J. W. Cornforth, F.R.S., and Professor G. Popják, F.R.S.

Radioassays. ^{14}C and ^3H counting was carried out in a Packard Tri-Carb Liquid Scintillation system, series 314E, with a Packard Automatic Control and Automatic Sample Changer, model 500D (Packard Instrument Co. Inc., La Grange, Ill., U.S.A.). The two isotopes were counted simultaneously and their respective radioactivities calculated by the 'discriminator ratio' method of Okita, Kabara, Richardson & Le Roy (1957). It was established that the degree of quenching caused by each of the compounds under investigation was negligible.

Detection of compounds on thin-layer chromatograms. Compounds were revealed under u.v. light, in the presence of Rhodamine 6G, as fluorescent or u.v.-absorbing spots or bands. Rhodamine was incorporated during the preparation of Kieselgel G plates, whereas AgNO₃-impregnated Kieselgel G plates were sprayed, after development, with a solution of Rhodamine 6G in acetone. Before being assayed for radioactivity, eluted samples were freed from any traces of Rhodamine by chromatography on columns of alumina (10g.; Brockmann grade III) with 10% (v/v) ether in light petroleum as eluent.

Melting points. All melting points are uncorrected.

Solvents. All solvents were redistilled before use. Light petroleum refers to the fraction boiling between 40 and 60°.

Preparation of labelled β -amyrin and β -amyrin acetate. The preparation of labelled β -amyrin and β -amyrin acetate from pea seedlings incubated with (3*RS*)-[2- ^{14}C , (4*R*)-4- $^3\text{H}_1$]MVA was described by Rees *et al.* (1966). The β -amyrin isolated contained approx. 16% of α -amyrin.

Degradation of β -amyrin. The sequence of reactions used to degrade the β -amyrin molecule is outlined in Scheme 1. Each reaction was carried out first on unlabelled β -amyrin, and the products were characterized by melting point, by u.v. and i.r. spectra and, in most cases, by preparation of derivatives. The labelled products were shown to be identical with these characterized products by thin-layer chromatography, by u.v. and i.r. spectra and by m.p. and mixed m.p.

Although the β -amyrin isolated from peas contained 16%

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† Abbreviation: MVA, mevalonic acid.

of α -amyryne (II), this should not be of significance in the degradation studies, since the labelled amyryne sample was diluted 150–200-fold with unlabelled β -amyryne, and this and the degradation products were rigorously purified by recrystallization. Further, because of the lowered reactivity of its double bond compared with that of β -amyryne, α -amyryne does not react under the conditions used to form β -amyradienediol acetate and β -amyradienol-II acetate from β -amyryne (Simonsen & Ross, 1957).

β -Amyrone (olean-12-en-3-one) (III). Labelled β -amyryne (21860 disintegrations/min. of ^{14}C) was mixed with carrier material (180 mg., 0.42 m-mole) and oxidized with chromic acid by the method of Brown & Garg (1961). The β -amyryne was dissolved in ether (10 ml.) and the theoretical amount of chromic acid (0.14 m-mole of $\text{Na}_2\text{Cr}_2\text{O}_7 \cdot 2\text{H}_2\text{O}$ and 0.80 m-mole of conc. H_2SO_4 diluted to 0.3 ml. with water) added dropwise at room temperature. The mixture was stirred by a magnetic stirrer for 4 hr., and then diluted with water (10 ml.) and extracted with ether (2×10 ml.). The combined ethereal extracts were washed with aq. NaHCO_3 soln. and water, dried over anhydrous Na_2SO_4 and evaporated to dryness. The product was chromatographed on a column of alumina (20 g.; Brockmann grade III), and elution with 6% (v/v) ether in light petroleum (200 ml.) yielded crude β -amyrone (189 mg.). A portion of this product (32 mg.) was purified from traces of unchanged β -amyryne by thin-layer chromatography on Kieselgel G with 3% (v/v) ethyl acetate in benzene as developing solvent. (R_F of β -amyryne, 0.21; R_F of β -amyrone, 0.52). The β -amyrone (31 mg.) was eluted and recrystallized several times each from methanol and aqueous ethanol, and had m.p. 179° [lit. m.p. 178 – 180° (Halsall & Alpin, 1964)], ν_{max} (Nujol) at 1720 cm^{-1} . The thoroughly dried sample was assayed for radioactivity.

Unlabelled β -amyrone (m.p. and mixed m.p. with radioactive sample 179°) prepared in the same way was further characterized by formation of its semicarbazone, which had, after four crystallizations from methanol, m.p. 247 – 249° [lit. m.p. 248 – 249° (Ruzicka & Wirz, 1941)].

β -Amyradienol-II acetate [3 β -acetoxyoleana-11(12),13(18)-diene] (IV). This was prepared by the method of Ruzicka, Müller & Schellenberg (1939b). Doubly labelled β -amyryne acetate (5470 disintegrations/min. of ^{14}C) diluted with unlabelled carrier material (150 mg.) was dissolved in boiling acetic acid (6 ml.), and a solution of selenium dioxide (75 mg.) in water (0.15 ml.) and acetic acid (6 ml.) added dropwise over 30 min. The boiling was continued for a further 30 min. and sodium acetate (800 mg.) was then added; after a further 15 min. boiling the hot mixture was filtered. The reaction product was precipitated from the

filtrate by the addition of water, collected by filtration and dried in a vacuum desiccator. The dried product was dissolved in ether and the solution washed successively with water, aq. NaHCO_3 soln. and water, dried over anhydrous Na_2SO_4 and evaporated to dryness. Chromatography of this product (160 mg.) on alumina (30 g.; Brockmann grade III) gave, on elution with 30% (v/v) ether in light petroleum (300 ml.), 155 mg. of crude β -amyradienol-II acetate. Further purification by thin-layer chromatography on Kieselgel G with 3% (v/v) ethyl acetate in benzene as developing solvent yielded 142 mg. of a substance with R_F 0.62. This material was freed from unchanged β -amyryne acetate by thin-layer chromatography on AgNO_3 -impregnated Kieselgel G plates, which were developed twice with 30% (v/v) benzene in light petroleum (R_F of β -amyryne acetate 0.43; R_F of β -amyradienol-II acetate 0.31). The eluted β -amyradienol-II acetate (137 mg.) was recrystallized four times from acetic acid to give plates, m.p. 227 – 228° [lit. m.p. 228 – 229° (Simonsen & Ross, 1957)], λ_{max} (ethanol) at 242 , 250 and $259\text{ m}\mu$ [lit. λ_{max} at 243 , 251 and $260\text{ m}\mu$ (Simonsen & Ross, 1957)]. The crystalline material (46 mg.) was used for radioassay.

Unlabelled β -amyradienol-II acetate was further characterized by saponification for 5 hr. by the standard method to give the free alcohol, which crystallized from ethanol as plates, m.p. 227 – 228° [lit. m.p. 228 – 229° (Simonsen & Ross, 1957)], mixed m.p. with the acetate 178 – 194° .

β -Amyranonol acetate (3 β -acetoxy-12-oxo-oleanane) (V). This was prepared by the method of Spring (1933). A solution of labelled β -amyryne acetate (5970 disintegrations/min. of ^{14}C) together with unlabelled carrier (120 mg.) in acetic acid (10.67 ml.) was heated for 2 hr. on a steam bath with Perhydrol (100 vol. H_2O_2) (0.307 ml.). After addition of water (30 ml.) the precipitated product was collected, dried in a vacuum desiccator and dissolved in ether (20 ml.). The ethereal solution was washed successively with aq. NaHCO_3 soln. and water, dried over anhydrous Na_2SO_4 and evaporated to dryness. The crude product (122 mg.) was chromatographed twice on Kieselgel G plates, which were developed with 3% (v/v) ethyl acetate in benzene. This system separated β -amyranonol acetate (93 mg.) from a u.v.-absorbing band of higher mobility. β -Amyranonol acetate crystallized from ethanol as plates, m.p. 292 – 294° [lit. m.p. 291 – 292° (Spring, 1933); 288 – 291° (Budziarek, Johnston, Manson & Spring, 1951); 292 – 293° (Ruzicka, Müller & Schellenberg, 1939a)], λ_{max} (ethanol) at $289\text{ m}\mu$ (weak band) [lit. λ_{max} at $280\text{ m}\mu$ (Simonsen & Ross, 1957), $290\text{ m}\mu$ (Ruzicka *et al.* 1939a)], ν_{max} (Nujol mull) at 1710 cm^{-1} . There was no i.r. band in the region 850 –

Scheme 1. $\bullet = {}^{14}\text{C}$; $\text{T} = {}^3\text{H}$; $\text{Ac} = \text{CH}_3 \cdot \text{CO}$.

790 cm^{-1} , indicating the absence of a trisubstituted double bond.

A sample of the crystalline material (38 mg.) was used for radioassay.

β -Amyrenonol acetate (3 β -acetoxy-11-oxo-olean-12-ene) (VI). This was prepared by the method of Ruzicka *et al.* (1939a). A solution of CrO_3 (88 mg.) in 80% (v/v) acetic acid (2 ml.) was added dropwise over 30 min. to a refluxing solution of doubly labelled β -amyrin acetate (4900 dis-

integrations/min. of ${}^{14}\text{C}$) together with unlabelled carrier (105 mg.) in acetic acid (7 ml.). After further refluxing for 1 hr., the reaction mixture was diluted with water (10 ml.) and extracted with ether (3×15 ml.). The combined ethereal extracts were freed from acid by successive washings with aq. NaHCO_3 soln. (twice) and water (twice), dried over anhydrous Na_2SO_4 and evaporated to dryness. The crude product was purified twice by thin-layer chromatography on Kieselgel G with 6% (v/v) ethyl acetate in

benzene as developing solvent. This separated β -amyrenol acetate (75 mg.; R_F 0.48) from unchanged β -amyrin acetate (R_F 0.70) and a minor product, β -amyranol acetate (R_F 0.44). The β -amyrenol acetate was recrystallized twice from ethanol as needles, m.p. 262–263° [lit. m.p. 264–265° (Ruzicka *et al.* 1939a)], $\lambda_{\max.}$ (ethanol) at 247 m μ [lit. $\lambda_{\max.}$ at 245 m μ (Ruzicka *et al.* 1939b; Beynon, Sharples & Spring, 1938)], $\nu_{\max.}$ (Nujol mull) at 1670 and 1625 cm.⁻¹. A sample (24 mg.) of the crystalline material was assayed for radioactivity.

β -Amyradienediol acetate [3-*acetoxy*-12,19-dioxo-oleana-9(11),13(18)-diene] (VII). This was prepared by the method of Ruzicka, Jeger & Norymberski (1942). A mixture of doubly labelled β -amyrin acetate (14100 disintegrations/min. of ¹⁴C) together with unlabelled carrier (100 mg.) and finely pulverized selenium dioxide (190 mg.) in peroxide-free dioxan (5.5 ml.) was heated in an evacuated sealed tube for 18 hr. at 210°. The reaction mixture was filtered and the filtrate evaporated to dryness under reduced pressure in a rotary film evaporator. The residue was dissolved in ether, and the ethereal solution washed with aq. NaHCO₃ soln. and water and evaporated to dryness. The crude product (116 mg.) was chromatographed on a column of alumina (30 g.; Brockmann grade III), and the product (90 mg.), which was eluted with ether, was further purified by thin-layer chromatography on Kieselgel G with 20% (v/v) ethyl acetate in benzene as developing solvent. The main compound (74 mg.; R_F 0.44) was recrystallized several times from aqueous methanol to give β -amyradienediol acetate as plates, m.p. 237–238° [lit. m.p. 239° (Ruzicka *et al.* 1942); 240° (Simonsen & Ross, 1957)], $\lambda_{\max.}$ (ethanol) at 280 m μ , inflexion at 360 m μ [lit. $\lambda_{\max.}$ at 280 m μ and inflexion at 360 m μ (Simonsen & Ross, 1957)], $\nu_{\max.}$ (Nujol mull) at 1670 and 1625 cm.⁻¹. The crystalline material (30 mg.) was assayed for radioactivity.

The unlabelled β -amyradienediol acetate was further characterized by saponification to the free alcohol, which was crystallized from chloroform–hexane, m.p. 287–289° [lit. m.p. 290–291° (Simonsen & Ross, 1957)].

Keto-enol tautomerism of β -amyranol acetate. Purified β -amyranol acetate (28 mg.; 1396 disintegrations/min. of ¹⁴C) was dissolved in ethanolic 12% (w/v) KOH (25 ml.), and aq. 50% (v/v) ethanol (25 ml.) was added. After refluxing for 6 days, the mixture was diluted with water (150 ml.) and extracted with ether (3 × 100 ml.). The ethereal solution was washed free from alkali with water, dried over Na₂SO₄ and evaporated to dryness. The crude product was purified by thin-layer chromatography on Kieselgel G, with chloroform as developing solvent. After elution with chloroform the β -amyranol (22 mg.; R_F 0.19) was assayed for radioactivity. The recovered β -amyranol was shown by thin-layer chromatography to be identical with an authentic sample obtained by saponification of unlabelled β -amyranol acetate.

RESULTS AND DISCUSSION

From theoretical considerations, Ruzicka's group (Eschenmoser *et al.* 1955) postulated that the biosynthesis of all triterpenes and related compounds could be explained by a number of schemes involving the folding of squalene in a well-defined conformation, followed by a series of concerted cyclization

reactions, each obeying normal stereochemical rules. The carbonium ion intermediate expected from such a cyclization could then undergo a series of 1,2- (Wagner–Meerwein) shifts, before being stabilized, usually by proton loss from some position in the molecule.

One such postulated mechanism is illustrated in Scheme 2, which shows the formation of β -amyrin by cyclization of squalene labelled from [2-¹⁴C,(4*R*)-4-³H₁]MVA. From this it can be seen that two labelled hydrogen atoms are involved in 1,2-hydride shifts, to produce an electron deficiency at C-13, which is neutralized by loss of a proton from C-12, to give the labelled β -amyrin (VIII). Therefore, if this concerted mechanism is functioning *in vivo*, the labelled hydrogen atoms in β -amyrin biosynthesized from [2-¹⁴C,(4*R*)-4-³H₁]MVA should be located at C-3, C-5, C-9, C-18 and C-19 (two). It had previously been shown (Rees *et al.* 1966) that six labelled hydrogen atoms were retained in β -amyrin biosynthesized from this substrate. In an extension of this work the series of degradative experiments outlined in Scheme 1 was carried out to verify that the labelled hydrogen atoms were located at the positions expected from the postulated mechanism.

The ³H/¹⁴C atomic ratios obtained for squalene and β -amyrin biosynthesized from [2-¹⁴C,(4*R*)-4-³H₁]MVA, and for the various degradation products, are shown in Table 1.

The ³H/¹⁴C atomic ratio obtained for β -amyrin is the same as that for squalene, 6:6, thus confirming our previous result. The ³H/¹⁴C atomic ratio 5:6 for β -amyrene (III) shows that one labelled hydrogen atom was lost in the formation of this derivative. The conversion of β -amyrin into β -amyrene involves only the oxidation of the secondary alcoholic group of β -amyrin to a keto group, with the loss of one hydrogen atom from C-3, and this hydrogen atom must therefore be labelled.

Allylic oxidation of β -amyrin acetate with chromium trioxide in acetic acid removes two hydrogen atoms from C-11 to form the keto compound β -amyrenol acetate (VI). Since the ³H/¹⁴C atomic ratio obtained for this product was the same as for the original β -amyrin acetate, 6:6, no tritium had been lost, showing that the hydrogen atoms at C-11 of β -amyrin are unlabelled.

The 12-oxo compound, β -amyranol acetate (V), was formed, also without loss of tritium, by oxidation of β -amyrin acetate with hydrogen peroxide in acetic acid. At first sight, it might be expected that this reaction would remove the hydrogen atom from C-12 of β -amyrin, but results obtained with β -amyrin labelled from [2-¹⁴C,(5*R*)-5-³H₁]MVA and [2-¹⁴C,5-³H₂]MVA (H. H. Rees, G. Britton & T. W. Goodwin, unpublished work) show that this is not so, since the hydrogen atom

Scheme 2. ● = ^{14}C ; T = ^3H .

migrates from C-12 to C-13. Therefore the formation of the 12-oxo compound does not give conclusive evidence for the absence of tritium from C-12. Enolization of the labelled β -amyranol acetate,

however, under conditions that have been shown to exchange all the hydrogen atoms at C-11 and C-13 (H. H. Rees, G. Britton & T. W. Goodwin, unpublished work), led to recovery of the starting

Table 1. $^3\text{H}/^{14}\text{C}$ ratios of various compounds formed by oxidative degradation of β -amyrin biosynthesized from $[2\text{-}^{14}\text{C}, (4R)\text{-}4\text{-}^3\text{H}_1]\text{MVA}$

Substance	Radioactivity (disintegrations/min.)		$^3\text{H}/^{14}\text{C}$ radioactivity ratio	Mean $^3\text{H}/^{14}\text{C}$ atomic ratio ($\pm$ S.E.M.) (based on β -amyrin)
	^3H	^{14}C		
$[2\text{-}^{14}\text{C}, (4R)\text{-}4\text{-}^3\text{H}_1]\text{MVA}$	120290	22696	5.300	
Squalene	15794	3109	5.080	
β -Amyrin	24622	4725	5.211	$6.16 \pm 0.04:6^*$
β -Amyrone	3414	778	4.388	$5.05 \pm 0.02:6$
β -Amyradienol-II acetate	8793	1945	4.521	$5.21 \pm 0.03:6$
β -Amyranonol acetate	9844	1904	5.170	$5.96 \pm 0.03:6$
Enolized β -amyranonol acetate	5550	1080	5.139	$5.92 \pm 0.03:6$
β -Amyrenonol acetate	6011	1139	5.277	$6.08 \pm 0.05:6$
β -Amyradienedionol acetate	5860	3728	1.572	$1.81 \pm 0.04:6$

* Based on squalene.

material without loss of tritium, thus showing that the hydrogen atom at C-12 of β -amyrin is not labelled.

β -Amyradienol-II acetate (IV), formed from β -amyrin acetate by the action of selenium dioxide in acetic acid, had a $^3\text{H}/^{14}\text{C}$ atomic ratio 5:6, showing a loss of one labelled hydrogen atom. This reaction would remove one hydrogen atom each from C-11 and C-18, and, since it has been shown that the hydrogen atom at C-11 is not labelled, the tritium lost must have been from C-18 of β -amyrin. The possible loss of the hydrogen atom from C-12 in this reaction, unlikely on mechanistic grounds, would not affect the result, since this hydrogen atom has been shown not to be labelled.

Oxidation of β -amyrin acetate with selenium dioxide in dioxan to give β -amyradienedionol acetate (VII) resulted in the loss of four labelled hydrogen atoms to give a product with a $^3\text{H}/^{14}\text{C}$ atomic ratio 2:6. According to the postulated mechanism (Beaton, Johnston, McKean & Spring, 1953), six hydrogen atoms are lost in the formation of this compound, one each from C-9, C-11, C-12 and C-18 and two from C-19. Since it has already been shown that the hydrogen atoms at C-11 and C-12 are not labelled, the remaining four hydrogen atoms, those at C-9, C-18 and C-19 (two) in β -amyrin, must be labelled.

The location has therefore been established of five of the six labelled hydrogen atoms in β -amyrin biosynthesized from $[2\text{-}^{14}\text{C}, (4R)\text{-}4\text{-}^3\text{H}_1]\text{MVA}$. Two of these are located at C-19 and one at each of C-3, C-9 and C-18. No experimental verification was obtained for the location in the β -amyrin molecule of the final labelled hydrogen atom. It is, however, impossible to derive any mechanism for the cyclization of squalene to form β -amyrin, with labelled hydrogen atoms in the positions established, that would result in the location of the final labelled

hydrogen atom at any position other than C-5. The establishment of the location of the five labelled hydrogen atoms is sufficient to define the mechanism of squalene cyclization.

The results obtained therefore verify that the pentacyclic triterpene β -amyrin is formed, in peas, by a concerted process involving the folding of squalene in a chair-chair-chair-boat conformation, a series of cyclizations and a series of 1,2- (Wagner-Meerwein) shifts, as illustrated in Scheme 2. Although this is the only case, outside the sterol field, in which the postulated mechanism for the biosynthesis of a triterpene has been verified, it seems reasonable to expect that the formation of other triterpenoid compounds does occur by similar concerted mechanisms, as proposed by Ruzicka's group.

Note added in proof. The recent demonstration that the cyclization of squalene is a two-stage process involving 2,3-oxidosqualene (Corey & Ortiz de Montellano, 1967) does not affect our arguments.

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