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THE STEREOSPECIFICITY OF BIOSYNTHESIS OF SQUALENE AND β-AMYRIN IN *PISUM SATIVUM*

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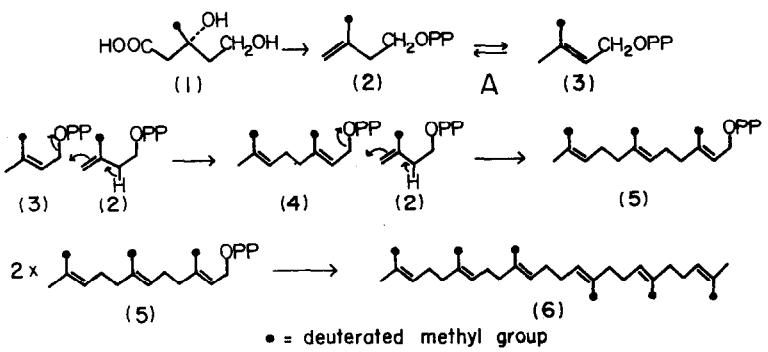
Key Word Index—*Pisum sativum*; Leguminosae; pea; biosynthesis; stereospecificity of enzymic reaction; triterpene; squalene; 2,3-oxidosqualene; β-amyrin; mevalonic acid-6,6,6-d_3; lanthanide shift reagent.

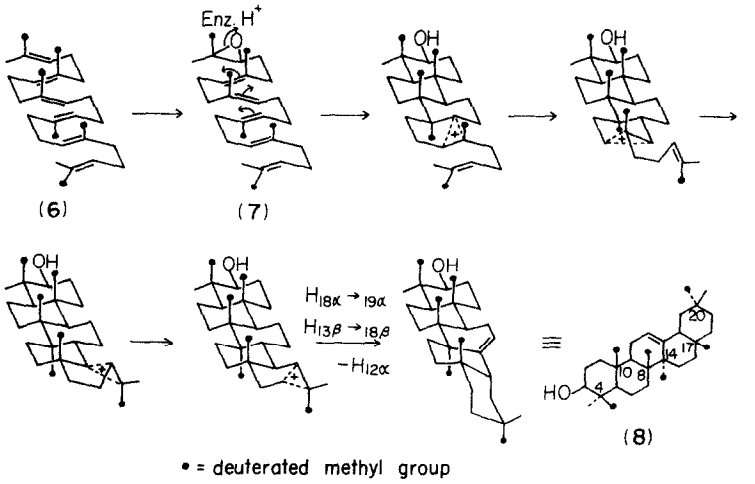
Abstract—The distribution of deuterium in squalene and β-amyrin, biosynthesized from mevalonic acid-6,6,6-d_3 in *Pisum sativum*, has been examined by NMR spectroscopy. It is demonstrated that the *cis*-terminal methyls of squalene and the 4β-, 8-, 10-, 14-, 17- and 20α-methyls of β-amyrin are generated stereospecifically from the 3-methyl group of mevalonic acid. Further, the high stereospecificity in the enzymic isomerization of isopentenyl pyrophosphate to dimethylallyl pyrophosphate and in the cyclization of squalene to β-amyrin has also been established. The formation of 2,3-oxidosqualene from MVA-[2-^{14}C] *via* squalene has been indicated by the time-course experiment of the biotransformation of this labelled tracer in a cell-free preparation from *P. sativum*.

INTRODUCTION

It has been documented[1] that the stereochemistry of the enzymic isomerization of isopentenyl pyrophosphate (IPP) (**2**) to dimethylallyl pyrophosphate (DMAPP) (**3**) is analogous to the subsequent stages[2], such as the condensation between IPP (**2**) and DMAPP (**3**) and IPP (**2**) and geranyl pyrophosphate (**4**), so that the *trans*-methyl of DMAPP (**3**) would be derived from the 3-methyl group of mevalonic acid (MVA) (**1**) as shown in Scheme 1. It has been suggested[3] that β-amyrin is formed from squalene by elimination of a proton from C-12 with simultaneous transfer of hydride ions from C-13 to C-18 and C-18 to C-19 as shown in Scheme 2. The evidence for the hydrogen migrations has been provided by the biosynthetic experiments using [2-^{14}C, (4*R*)-4-^{3}H$_1$]-MVA as a precursor[4, 5]. However, the stereospecific biosynthesis of the rings A and E of pentacyclic triterpenes from squalene (**6**) still remains experimentally unproved[6]. Biogenetically[3], in the formation of β-amyrin (**8**) from squalene (**6**), it is expected that the methyls at 4α and 20β are



Scheme 1. The stereospecific course of the biosynthesis of squalene (**6**) from MVA-6,6,6-d_3 (**1**).



Scheme 2. Stereospecific cyclization of squalene (**6**) to β-amyrin (**8**) via 2,3-oxidosqualene (**7**).

derived from the *trans*-terminal methyls and the methyls at 4β and 20α from the *cis*-terminal methyls. The establishment of this stereospecificity can be expected to furnish direct support for the hypothetical mechanism proposed for the cyclization of squalene (**6**) to β-amyrin (**8**) and other triterpenes[3, 6]. In order to prove these stereochemical relationships in the formation of squalene (**6**) and β-amyrin (**8**) in living tissues, the distribution of deuterium in **6** and **8** biosynthesized from MVA-6,6,6-d_3 (**1**) by germinating seeds of *Pisum sativum* has been examined by NMR spectroscopy. This spectroscopic procedure solves the problem of distinguishing chemically between a ^{14}C-labelled and an unlabelled methyl group in the two terminal methyls of squalene (**6**) and in the methyls at C-4 and C-20 of β-amyrin (**8**). We have reported the results in the form of preliminary communications[7]. Recently there appeared a brief paper reporting a NMR spectral method for differentiating between the 20α and 20β methyl groups of β-amyrin (**8**)[8]. This prompted us to report here our detailed results.

RESULTS AND DISCUSSION

For this triterpene biosynthesis, first a deuterium-labelled precursor, D,L-MVA-6,6,6-d_3 (**1**), was prepared as follows. Tetradeuteroacetic acid methyl ester was treated with allylmagnesium bromide to yield 4-trideuteromethyl-1,6-heptadien-4-ol, which on ozonization and subsequent oxidation afforded 3-hydroxy-3-trideuteromethylglutaric acid. The dimethyl ester of this acid was then reduced in two stages, by $LiAlH_4$ and $NaBH_4$, to yield MVA (**1**). The extent of deuteration of 3-methyl group of MVA (**1**) was above 99%. Squalene (**6**) and β-amyrin (**8**) were biosynthesized from MVA-6,6,6-d_3 (**1**) in germinating seeds of *P. sativum*, by incubation for 5 days.

The MS spectrum of the biosynthetically deuterated squalene (**6**) showed a molecular ion peak at *m/e* 428 and a base peak at *m/e* 72 instead of the *m/e* 410 and 69 of normal squalene. Thus, the biosynthesized squalene (**6**) contains eighteen deuterium atoms and one isoprene unit at the terminal end of the molecule with a fully deuterated methyl group.

The NMR spectrum of normal squalene (Fig. 1a) exhibited signals at δ 1·58 and 1·67 ppm due to methyl groups attached to the double bonds. The former corresponds to the resonance of protons of two terminal and four internal *cis*-methyls and the latter to terminal *trans*-methyls, respectively[9]. The signals of methylenes and vinyl protons were observed around δ 2·0 and 5·1 ppm respectively. However, the biosynthetically deuterated squalene (**6**) exhibited a similar spectrum, except for a peak at δ 1·58 ppm with a peak intensity less than 5% as compared to that of normal squalene. This demonstrated that the *cis*-terminal and the internal methyls of squalene (**6**) were stereoselectively deuterated as shown in Scheme 1. Further this also established that the *cis*-

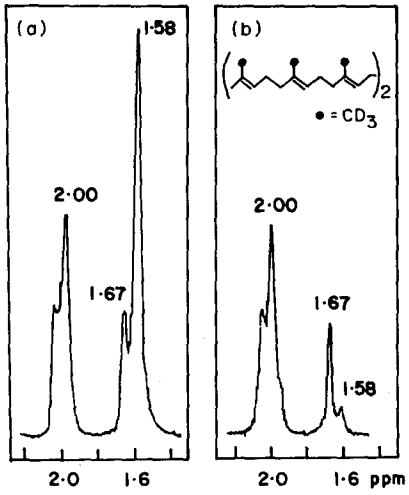


Fig. 1. Segments of NMR spectra of (a) normal and (b) biosynthetically deuterated squalenes.

terminal methyl groups of squalene (**6**) were stereospecifically derived from the 3-methyl group of MVA (**1**) *via* the methyl group of IPP (**2**) and that the methyl group of **2** was retained at the *cis*-position of the CH_2OPP group of DMAPP (**3**) in the reversible enzymic isomerization between **2** and **3** as shown in the stage A of Scheme 1. Moreover, the stereochemistry of this isomerization in the plant tissue was also established to be the same as in mammalian tissue[10].

The MS spectra of normal and the biosynthetically deuterated β-amyrins (**8**) are shown in Fig. 2. According to the mass spectral fragmentation pattern of Δ^{12}-oleanene[11, 12], the prominent peaks of the normal β-amyrin in the high mass region were assigned as described in Fig. 2a. The MS spectrum of the biosynthetically deuterated β-amyrin (**8**) showed a molecular ion at *m/e* 444 and the characteristic fragment ions at *m/e* 268, 227, 212 and 209. The *m/e* 268 corresponds to the the *m/e* 257 of normal β-amyrin. The *m/e* 227 fragment (base peak) corresponds to the intense and diagnostically important peak (*m/e* 218) of the normal species, resulting from a retro-Diels–Alder decomposition involving cleavages of the 9–10 and 8–14 bonds in the molecular ion[11]. This ion suffers further fragmentation losing either 18 mass units due to the CD_3 group or 15 mass units due to the Me group, yielding fragment ions of *m/e* 209 and 212 respectively[12]. The ions appearing at *m/e* 426, 218 and 203 (Fig. 2b) may be ascribed to the ions resulted from endogenous normal β-amyrin. Thus, on the basis of the MS fragmentation pattern it was found that the biosynthesized β-amyrin (**8**) possesses 6 CD_3 groups and that 3 of the 4 methyl groups attached to the rings D and E are CD_3 groups.

The NMR spectrum of biosynthetically deuterated β-amyrin (**8**) (Fig. 3b) showed the strong

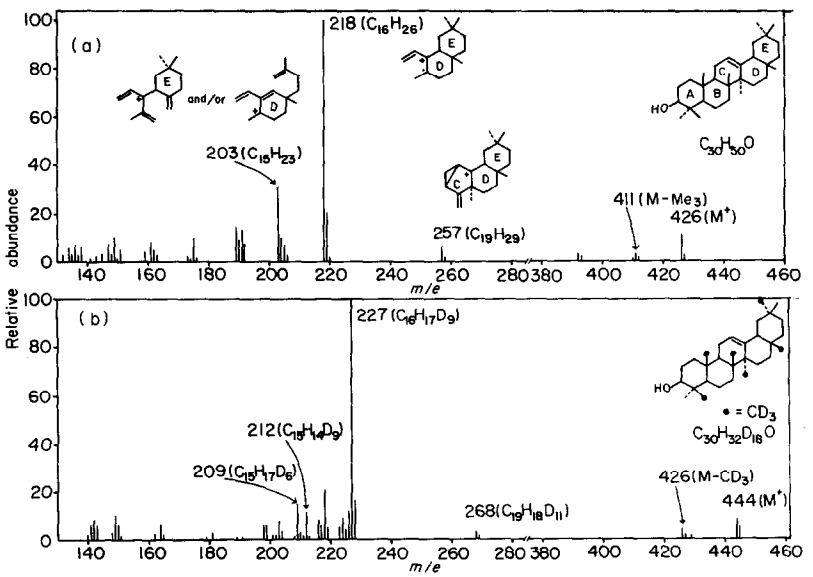


Fig. 2. MS spectra (a) normal and (b) biosynthetically deuterated β-amyrins.

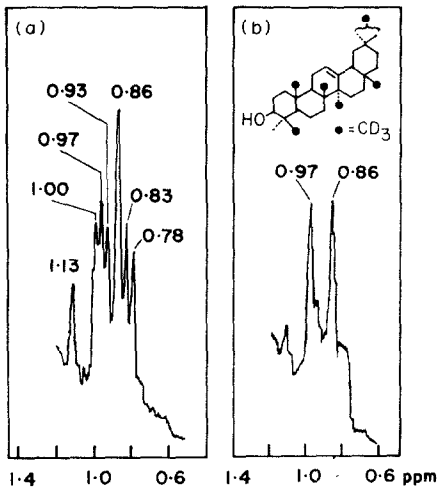


Fig. 3. Segments of NMR spectra of (a) normal and (b) biosynthetically deuterated β-amyrins.

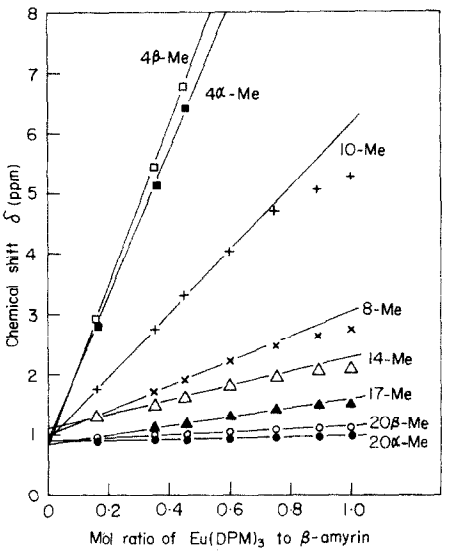


Fig. 4. Variation in the chemical shift for the methyl protons of β-amyrin in $CDCl_3$ with increasing concentration of $Eu(DPM)_3$.

suppression of peaks at δ 0·78 (4β-Me), 0·83 (17-Me), 0·93 (10-Me), 1·00 (8-Me) and 1·13 ppm (14-Me), and a peak of half the height at δ 0·86 (20α and 20β-Me), as compared to that of normal β-amyrin (Fig. 3a)[13]. This suggests the following two points: (i) the pattern of the deuteration of methyl groups of β-amyrin (**8**) is as shown in Fig. 3b and (ii) the derivation of the 4β-methyl group of β-amyrin (**8**) from the 3-methyl of MVA (**1**), *via* the *cis*-terminal methyl of squalene (**6**).

However, the NMR spectroscopic examination of the deuterium distribution in the 20α- and 20β-methyls of the biosynthesized β-amyrin (**8**) was unsuccessful because of the overlapping of proton signals from the methyls in question (Fig. 3b). But when the NMR shift reagent[14], tris(dipivaloylmethanato) europium, $Eu(DPM)_3$, was utilized to resolve the problem, it was observed that all methyl signals of normal β-amyrin in $CDCl_3$ were shifted linearly with the amounts of $Eu(DPM)_3$ up to 0·8 mol equivalent (Fig. 4), and with one mol, there was a distinct resolution of all the methyl signals (Fig. 5a). It has been suggested that the co-ordinated Eu ion approaches the hydroxyl oxygen atoms from the direction of the C–O bond[15, 16]. This indicates that the order of proximity of methyl groups to the co-ordination site is 4β, 4α, 10, 8, 14, 17, 20β and 20α. In accordance with spatial proximity, signals were assigned to 8 methyls such that the order of their appearance varies from low to high field[15]. These assignments are consistent with those obtained by the method of internuclear double resonance[17] and of the specific deuteration[8]. The NMR spectrum of biosynthetically deuterated β-amyrin (**8**) in $CDCl_3$ solution containing 1 mol equivalent of $Eu(DPM)_3$ is shown in Fig. 5b. Only 2 methyl signals were observed at δ 11·23 and 1·08 ppm, which were assigned to the methyls of 4α and 20β respectively by a comparison with the spectrum of normal β-amyrin (Fig. 5a). The NMR spectrum of the mixture of normal and deuterated β-amyrins (**8**) was also measured in $CDCl_3$ containing 0·4 mol equivalent of $Eu(DPM)_3$. Further the intensity of the signals due to 4β, 10-, 8-, 14-, 17- and 20α-methyls was equal to nearly half that of 4α- and 20β-methyls (Fig. 5c). Moreover, the fact that the 4α-methyl group was not deuterated agreed with the result from the ordinary NMR measurements without the use of $Eu(DPM)_3$ described above. This suggested that the 4β-, 8-, 10-, 14-, 17- and 20α-methyls were fully deuterated and were derived stereospecifically from the 3-methyl group of MVA (**1**) *via* the *cis*-methyls of squalene (**6**).

The formation of 2,3-oxidosqualene (**7**) from squalene (**6**)[18] and its cyclization to

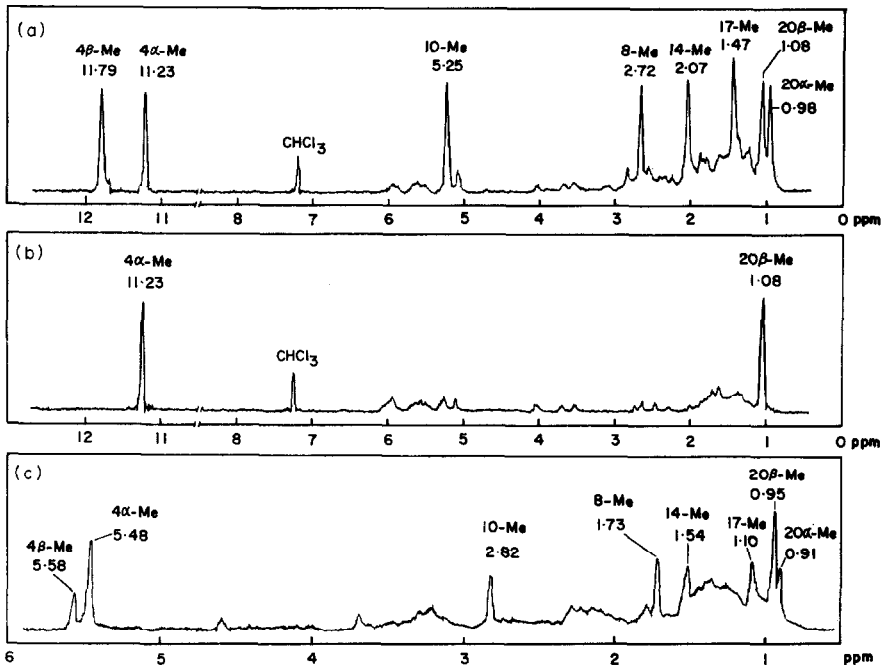


Fig. 5. NMR spectra of (a) normal and (b) biosynthetically deuterated β-amyrins in $CDCl_3$ containing 1 mol equivalent of $Eu(DPM)_3$ and of (c) a mixture of normal (3·49 mg) and deuterated β-amyrins (3·09 mg) containing 0·4 mol equivalent of $Eu(DPM)_3$.

lanosterol[19, 20] have been demonstrated in the mammalian enzyme system. The formation of the oxide (7) in the enzyme preparation of higher plants has not been proved, though its cyclization to β-amyrin (8) has been clarified[21]. The formation of 2,3-oxidosqualene (7) from MVA-[2-^{14}C] has now been examined by the time-course experiment in a cell-free preparation from *P. sativum*. The MVA-[2-^{14}C] was aerobically incubated with a 14000x *g* supernatant of the homogenate of the germinating pea seeds. Figure 6 shows the thin layer radiochromatograms of a product obtained from the incubation at such time intervals as shown in the figure. The radioactivity of squalene (6) increased with time of the incubation and reached a maximum after 3 hr, and the radioactivity was observed first in 2,3-oxidosqualene (7) (peak II in Fig. 6), as well as in a triterpene alcohol and a sterol fraction (peaks III and IV) containing principally β-amyrin and sitosterol, respectively. The radiochromatogram of a product obtained from the 24 hr-incubation was identical with that of the 3 hr-incubation product, and it was omitted from

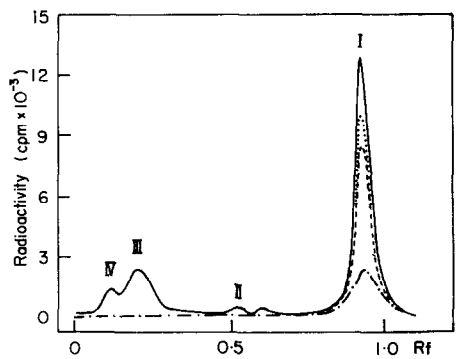


Fig. 6. Thin layer radiochromatograms of products from the incubations of MVA-[2-^{14}C] with a cell-free preparation of germinating seeds of *Pisum sativum*. Incubation time: —·— 10 min; ---- 30 min; ····· 1 hr; —— 3 hr. Peaks: I squalene (6), II 2,3-oxidosqualene (7), III triterpene alcohols (mainly β-amyrin (8)), IV sterols (mainly sitosterol). TLC was performed on the plate of silica gel G with *n*-hexane —— Et_2O (9:1).

Fig. 6. These facts indicate the enzymic formation of 2,3-oxidosqualene from MVA in this higher plant and the intermediacy of 2,3-oxidosqualene in the transformation of squalene to cyclic triterpenes.

It has been documented that squalene (**6**) is oxidized to (3*S*)-2,3-oxidosqualene (**7**) by squalene epoxydase without any inversion of the terminal *gem*-dimethyl group [22, 23, 24]. Assuming the oxide (**7**) to be cyclized in the chair-folding form as shown in Scheme 2, then, both the 4β- and 20α-methyl groups at 4- and 20-positions of β-amyrin (**8**) can be expected to be deuterated stereospecifically. It was observed that the deuterium-labeling patterns observed for β-amyrin (**8**) agree with those expected, suggesting that the cyclization process proceeds with high stereospecificity. Further it also provided experimental support for the proposal of Ruzicka and co-workers [3, 6] for the biogenesis of pentacyclic triterpenes having the *gem*-dimethyl groups in rings A and E. Moreover, these stereochemical relationships in the enzymic formation of squalene (**6**) and its cyclization to β-amyrin (**8**) in this higher plant are also in agreement with those documented for the biosynthesis of squalene and lanosterol in the mammalian enzyme system [22, 25].

EXPERIMENTAL

MS analyses were performed with a direct inlet system, ionizing at the order of 80 eV. NMR measurements were made in $CDCl_3$; NMR of β-amyrin in the presence of a lanthanide shift reagent was measured in $CDCl_3$ solns with concn ranging from 0 to 1 mol equiv. of Eu(DPM)$_3$. The chemical shift of signals are given in δ with tetramethylsilane as the internal standard.

Preparation of MVA-6,6,6-d$_3$ (**1**). Tetradeuteroacetic acid (deuteration degree not less than 99%) was methylated with ethereal CH_2N_2 and dried over Drierite and the mixture was conc to about 50 ml by fractional distillation. To a mixture of Mg ribbon (16 g), dry Et_2O (20 ml) and tetrahydrofuran (20 ml), was added a soln of dry Et_2O (80 ml) and tetrahydrofuran (160 ml) containing allyl bromide (64 g) and MeOAc prepared as above, with a gentle reflux. After refluxing for 2 hr further, the usual treatment of the reaction mixture gave 4-trideuteromethyl-1,6-heptadien-4-ol (15·8 g); ν_{max} (neat) 3400, 1645, 908 cm^{-1}; δ ppm ($CDCl_3$) 2·22 (*d*, *J* 6·6 Hz, 4H, allylic CH_2), 4·9–6·3 (*m*, 6H, vinyl group). A soln of this dienol (7·0 g) in CH_2Cl_2 (100 ml) and HOAc (10 ml) was ozonized at −70° until the soln became blue. After addition of HOAc (100 ml) CH_2Cl_2 was removed under red pres and the product was refluxed with 30% H_2O_2 (40 ml) for 8 hr. Removal of the solvent under red pres yielded a viscous oil which crystallized with time. Recrystallization from C_6H_6–dioxane (1:1) afforded 3-hydroxy-3-trideuteromethylglutaric acid (7·0 g, mp 108–109°, lit. [26] 110° for non-deuterated specimen); ν_{max} (KBr) 3400, 1710 cm^{-1}; δ ppm (D_2O) 2·72 (*s*, 4H, CH_2). (Found: C, 43·63; H + D, 7·93. Calc. for $C_6H_7D_3O_5$: C, 43·75; H + D, 7·93.). This acid (3·0 g) was methylated with CH_2N_2 to dimethyl 3-hydroxy-3-trideuteromethylglutarate. ν_{max} (neat) 3400, 1720 cm^{-1}; δ ppm ($CDCl_3$) 2·66 (*s*, 4H, CH_2), 3·70 (*s*, 6H, Me). To a soln of this ester (1·91 g) in dry Et_2O (20 ml) and tetrahydrofuran (20 ml) was added a suspension of $LiAlH_4$ (200 mg) in dry Et_2O (20 ml) with stirring at 0° during 1 hr, and the reaction mixture was stirred for an additional 10 min. Then, an aq soln (40 ml) of $NaBH_4$ (100 mg) was added to the above mixture at 0°, and stirred for 2 hr. $Ba(OH)_2$ soln (0·25N, 40 ml) was then added to the soln and stirred for 12 hr. The organic solvent was removed at red pres and the acid was liberated by the addition of 3N–H_2SO_4 (15 ml). The mixture was then saturated with $(NH_4)_2SO_4$ and extracted continuously with Et_2O. After drying, Et_2O was removed to yield a viscous oil, which was column chromatographed over Si gel (150 g) and eluted with mixtures of C_6H_6 and Me_2CO in increasing proportions. The eluate of C_6H_6–Me_2CO (1:1) contained MVA-6,6,6-d_3 (**1**), recrystallization of which from Me_2CO–Et_2O (1:1) at −78° afforded 520 mg of pure **1** (mp 27–28°, lit. [26, 27] 27° for non-deuterated specimen). ν_{max} (neat) 3450, 1730 cm^{-1}; δ ppm ($CDCl_3$) 1·89 (*dd*, 2H, C-4 CH_2), 2·54 and 2·58 (each *s*, 2H, C-2 CH_2), 3·6 (*s*, OH), 4·5 (*m*, 2H, C-5 CH_2). *N,N*-Dibenzylethylenediamine salt of MVA-6,6,6-d_3: mp 124–125°, lit. [26, 27] 125° for non-deuterated specimen. (Found: C, 62·20; H + D, 9·15; N, 5·39. Calc. for $C_{28}H_{38}D_6N_2O_8$: C, 62·03; H + D, 9·29; N, 5·18).

Incubation of MVA-6,6,6-d$_3$ with peas. MVA-6,6,6-d_3 (**1**) (555 mg) was made slightly alkaline with a dil. aq NaOH soln and accurately neutralized with 0·1N–HCl. The soln then made up to 50 ml with H_2O and dry peas, *P. sativum* (Japanese name, usui-endo), (50 g) was added [28]. This was incubated at 25°, and when the soln of MVA was completely absorbed, it was moistened with H_2O. After 5 days, the germinating seeds were ground in a mortar and extracted continuously with Me_2CO in a Soxhlet for 1 day. This extract on evaporation yielded a residue which was hydrolyzed by refluxing with 5% ethanolic KOH (30 ml) for 3 hr and EtOH was removed under red pres. The product was partitioned between Et_2O and H_2O (3:1, 160 ml), and the aq layer re-extracted with Et_2O. The combined ethereal soln was washed with H_2O and on evaporation afforded a semi-solid material (397 mg) which was separated by preparative TLC [Si gel G, *n*-hexane–EtOAc (2:1)] into 3 fractions: hydrocarbons (30 mg), triterpene alcohols (28 mg) and sterols (280 mg). The hydrocarbon fraction was purified by repeated TLC (Si gel $HF_{254+366}$, *n*-hexane) to give squalene (**6**) (6 mg): *m/e* 428 (M^+, 4%), 410 (2·5), 382 (3), 356 (4·5), 143 (21·5), 111 (14), 97 (28), 84 (55), 72 (base), 57 (73). The NMR spectrum in $CDCl_3$ is given in Fig. 1b. The triterpene alcohol fraction contained mainly β-amyrin (**8**), which was purified by TLC [Si gel GF, *n*-hexane–EtOAc (3:1)] and recrystallized (15 mg; mp and mixed mp 199–201°). GLC analysis with a 2% SE-30 column showed this sample of β-amyrin to be contaminated with 10% of α-amyrin. The MS spectrum of the biosynthesized β-amyrin is shown in Fig. 2 together with that of normal β-amyrin. The NMR spectra in $CDCl_3$ are shown in Figs. 3b and 5b. The sterol fraction was further purified by TLC and recrystallized to yield sitosterol (180 mg; mp and mixed mp 127–128°). Purity of the sample was checked by GLC analysis with a 2% SE-30 column to be above 95%. NMR and MS spectra of the biosynthesized sitosterol were identical with those of the authentic specimen and the detectable extent of deuteration was not observed on these spectra.

Time-course of the incorporation of MVA-[2-^{14}C] into triterpenes in the cell-free preparation of peas. Peas, *P. sativum*, (100 g) germinated at room temp. for 24 hr were macerated at 0° in a Waring Blender for 90 sec with 0·1 M Pi buffer (250 ml, pH 7·4) containing 0·45 M sucrose and 0·01 M $MgSO_4$ [29] and the resulting slurry on filtration through

cheesecloth yielded a homogenate. The homogenate was centrifuged at 14000*g* for 20 min at 0°. The supernatant (20 ml) was incubated with the *N,N*-dibenzylethylenediamine salt of D,L-MVA-[2-^{14}C] ($2{\cdot}22 \times 10^6$ cpm, 0·03 μmol) and disodium salt of ATP (5 mg) at 25° in air. Incubations were carried out for 10 min, 30 min, 1 hr, 3 hr and 24 hr and were terminated by the addition of EtOH. The mixtures were hydrolyzed by refluxing with 5% ethanolic KOH (20 ml) for 2 hr. It was then diluted with H_2O and extracted with *n*-hexane. After removal of solvent, the product were diluted to 0·1 ml with *n*-hexane. Aliquots (10 μl) of the soln of products were analyzed by radio-TLC on a Si gel G plate with *n*-hexane–Et_2O (9:1). The results are given in Fig. 6. The product obtained from the 24 hr incubation was fractionated into 3 fractions, squalene (**6**) (peak I), 2,3-oxidosqualene (7) (peak II) and a mixture of triterpene alcohols and sterols (peak III and IV), by preparative TLC (Si gel G, *n*-hexane–Et_2O). The squalene fraction was purified by TLC (Si gel $HF_{254+366}$, *n*-hexane). 2,3-Oxidosqualene isolated was diluted with an authentic sample (90 mg) prepared from squalene and converted to 2,3-diol by treatment with perchloric acid[30]. This diol was purified by TLC [Si gel $HF_{254+366}$, *n*-hexane–EtOAc (7:3)]. The fraction of a mixture of triterpene alcohols and sterols was further separated by TLC [Si gel GF, *n*-hexane–EtOAc (3:1)]. These fractions were subjected to GLC with a 2% SE-30 column and TLC analyses [Si gel GF, *n*-hexane–EtOAc (3:1)] and found to be principally composed of β-amyrin and sitosterol, respectively. The radioactivity of the compounds was assayed as previously described[31]. Squalene (**6**), 2,3-oxidosqualene (7), a triterpene alcohol fraction and a sterol fraction had activities $1{\cdot}00 \times 10^5$, $2{\cdot}58 \times 10^2$, $2{\cdot}04 \times 10^4$ and $9{\cdot}13 \times 10^2$ cpm, respectively.

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