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THE BIOSYNTHESIS OF 31-NORCYCLOLAUDENONE IN *MUSA SAPIENTUM*

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Abstract—The biosynthesis of 31-norcyclolaudenone has been investigated in banana peel (*Musa sapientum* L.) using $^{14}CO_2$, 2-^{14}C-acetic acid, 2-^{14}C-mevalonic acid and 2-^{14}C-(4R)-4-$^{3}H_1$-mevalonic acid. For incubation periods up to 6 days, carbon dioxide was the most effective precursor. Incubations with the doubly labeled mevalonic acid demonstrated initial removal of the 4α-methyl group of the 4,4-dimethyl triterpene precursor in the biosynthesis of 31-norcyclolaudenone. A mechanism has been proposed that is compatible with net inversion of the original 4β-methyl group to the 4α-position during the formation of this 4α-monomethyl-3-ketone. In addition, these studies have indicated that the C-24 hydrogen atom is retained during the C-24 methylation process which forms the 24-methyl-25(26)-en side chain. The hydrogen atom is presumably retained at C-24.

INTRODUCTION

STUDIES of the triterpene and sterol components of banana peel demonstrated the presence of large quantities of an unidentified triterpene ketone.[1] This ketone has recently been shown to be 31-norcyclolaudenone (4α,14α,24β-trimethyl-13βCH$_3$,17αH,20αH-9,19-cyclocholest-25-en-3-one).[2] The formation of this triterpene presents several interesting problems. Few 9,19-cyclopropane triterpenes have been reported to contain a C-25(26)-en side chain. These include cyclolaudenol (24β-methyl-9,19-cyclolanost-25-en-3β-ol),[3,4] and cycloart-25-en-3β-ol-24-one.[5] The formation of the 24-methyl side chain is important with respect to the fate of the C-24 hydrogen atom upon alkylation at this position with subsequent formation of the C-25(26) double bond. The biosynthesis of the phytosterol side chain proceeds with retention of the C-24 hydrogen atom, probably by migration to C-25.[6–9]

Secondly, the sequence of removal of the 4α- and 4β-methyl groups from 4,4-dimethyl-triterpenes is of interest. Early studies of cholesterol formation in mammalian tissue indicated the initial removal of the 4β-methyl group of lanosterol in the biosynthesis of the 4α-monomethyl sterol precursor.[10] There was originally no reason to question these

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findings since from structural analogies one would expect the 4β-methyl group to be removed to form the 4α-monomethyl product. More recent experiments, however, have indicated that the opposite case occurs with the initial removal of the 4α-methyl group.[11] This is an unprecedented result and implies net inversion of the 4β-methyl group during the first demethylation process. Recent investigations have shown the same situation to occur during the conversion of cycloartanol to 31-norcycloartanol in *Polypodium vulgare*,[12] and 24-methylenecycloartanol to cycloeucalenol in *M. sapientum*.[13] The investigations described in this communication demonstrate that this mechanism also operates during the formation of 31-norcyclolaudenone in *M. sapientum*.

RESULTS AND DISCUSSION

The results of the labeling experiments with a number of ^{14}C-substrates are summarized in Table 1. With 2-^{14}C-mevalonic acid and 2-^{14}C-acetic acid the per cent incorporation

TABLE 1. INCORPORATION OF 2-^{14}C-MEVALONATE, 2-^{14}C-ACETATE AND $^{14}CO_2$ INTO 31-NORCYCLOLAUDENONE

Substrate	Incubation period	Total dis./min incorporated into neutral lipid	31-Norcyclolaudenone Cryst.	mg	dis./min	% of total incorporation	dis./min/mg
2-^{14}C-Mevalonate DBED salt 5 μc	24 hr-tissue slice	$7{\cdot}4 \times 10^5$	1st	38	190	0·0004	5
	3 days-whole tissue	$3{\cdot}4 \times 10^5$	1st	32	2080	0·006	65
	6 days-whole tissue	$5{\cdot}8 \times 10^5$	1st	38	2584	0·005	68
2-^{14}C-Acetate 30 μc sodium salt	24 hr-tissue slice	$3{\cdot}8 \times 10^6$	1st	41	500	0·0001	12
	3 days-whole tissue	$3{\cdot}2 \times 10^5$	1st	36	2016	0·006	56
	6 days-whole tissue	$4{\cdot}6 \times 10^5$	1st	42	3990	0·006	95
$Ba^{14}CO_3$ 1 mc			1st	50	8640	0·03	17·3
	48 hr-tissue slice	$2{\cdot}6 \times 10^5$	3rd	39	6160	—	158
In the presence of H_2SO_4			5th	25	4080	—	163
			7th	13	2080	—	160

into 31-norcyclolaudenone was low even after 6 days. It is important to note that, although incorporation of label into this triterpene was negligible, significant amounts of label were incorporated into other triterpenes in this tissue, even after much shorter incubation periods.[13] The formation of 31-norcyclolaudenone, therefore, appears to be independent of the biosynthesis of the other triterpenes in this tissue. The inability to effectively label 31-norcyclolaudenone from these substrates could reflect either an extremely slow turnover or a highly inaccessible pool of mevalonic acid or some other early intermediate for the formation of this particular triterpene. Carbon dioxide proved an effective precursor of 31-norcyclolaudenone. The per cent incorporation of $^{14}CO_2$ after 48 hr was five times that after incubation for 6 days with either 2-^{14}C-mevalonic acid or 2-^{14}C-acetic acid.

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Incubations were also performed with 2-^{14}C-(4R)-4-$^{3}H_1$-mevalonic acid. Squalene formed from this substrate is labeled as indicated in Fig. 1.[9] The stereochemistry of the tritium atoms are not indicated in this figure. Calculations are based on a ^{3}H–^{14}C atomic ratio of 6:6 for squalene labeled in this manner. There is no loss of either label upon conversion to cycloartenol and 24-methylenecycloartanol.[9,13] Alkylation of cycloartenol at

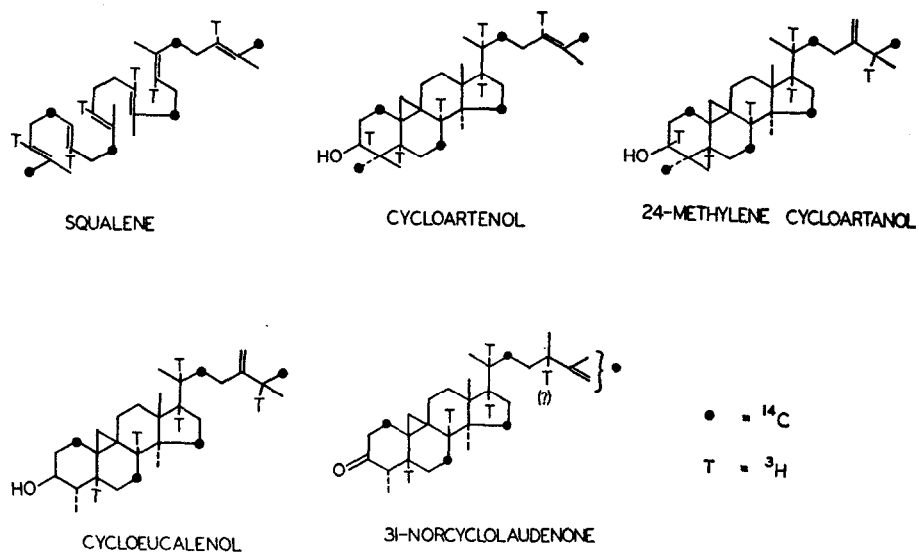

FIG. 1.

C-24 to form 24-methylenecycloartanol thus proceeds with retention of the C-24 tritium atom. It is probably retained by migration to C-25 as indicated in Fig. 3A.[9,14–17] Cycloeucalenol isolated from these incubations of banana peel had the same ^{3}H–^{14}C radioactivity ratio as squalene.[13] Oxidation to cycloeucalenone failed to change this ratio, indicating the absence of tritium at C-3.

The ^{3}H–^{14}C radioactivity ratio of 31-norcyclolaudenone was also the same as in squalene (Table 2). There was obviously no tritium present at C-3 and therefore one atomic equivalent of ^{14}C must have been lost from the 4,4-dimethyltriterpene precursor. The radioactivity must have been associated with the C-4 methyl group that was lost since this was the only carbon atom removed in this transformation. The ^{3}H–^{14}C atomic ratio of 31-norcyclolaudenone relative to squalene must therefore be 5:5. If the 4β-methyl group of the 4,4-dimethyl precursor were labeled exclusively with ^{14}C and was specifically removed during the demethylation process, the ratio of 5:5 would be expected. However, for those cyclic terpenes labeled from 2-^{14}C-mevalonic acid that have been degraded (soyasapogenol A, rosenonolactone), the 4α-methyl group is specifically labeled from this substrate.[18]

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TABLE 2. INCORPORATION OF 2-^{14}C-(4R)-4-$^{3}H_1$-MEVALONIC ACID INTO 31-NORCYCLOLAUDENONE

		Squalene	31-Norcyclolaudenone
^{3}H–^{14}C	Radioactivity Ratio		
	Experiment 1	4·10	4·09
	Experiment 2	5·95	6·23
	Experiment 3	32·22	32·74
	Experiment 4	31·96	31·65
^{3}H–^{14}C	Atomic Ratio		
	Experiment 1	—	4·98:5
	Experiment 2	—	5·28:5
	Experiment 3	—	5·02:5
	Experiment 4	—	5·05:5
Theoretical		6:6	5:5

5 g slices of banana peel were incubated for 24 hr with 12·5 μc of (4R)-$^{3}H_1$-MVA and with between 1 μc (Experiment 1) and 5 μc (Experiments 3 and 4) of 2-^{14}C-mevalonic acid.

The same is true of cycloartanol,[12] lanosterol[19] and lupeol formed from 2-^{14}C-mevalonic acid.[20] It thus seems probable to assume that the 4α-methyl group of the 4,4-dimethyl-triterpene precursor of 31-norcyclolaudenone was labeled in a similar manner from this substrate. From the experimental ^{3}H–^{14}C atomic ratio of 5:5 it can now be inferred that the 4α-methyl group of the precursor was lost during the demethylation process and that the original 4β-methyl group assumed the 4α-orientation in 31-norcyclolaudenone. Other workers have now shown that 31-norcyclolaudenol biosynthesized in *P. vulgare* has a ^{3}H–^{14}C atomic ratio of 5:5 based on a ratio of 6:6 for squalene.[12]

There is thus a similarity between C-4 demethylation of triterpenes in plant and animal tissues. That a 3-ketone is formed at some stage of this process has been demonstrated during lanosterol demethylation.[21,22] During the biosynthesis of β-sitosterol a 3-ketone is also formed at some stage of the biosynthetic sequence, presumably during the demethylation reaction.[6,8,9] It has now been demonstrated that this occurs during the conversion of 24-methylenecycloartanol to cycloeucalenol.[13] Any mechanism proposed to explain the first demethylation process must account for (1) the formation of a 3-ketone during this transformation, and (2) net inversion of the original 4β-methyl group to the 4α position.

Since the trans A/B ring juncture of 9,19-cyclopropane triterpenes represents a very rigid system, net inversion of the 4β-methyl group is best explained by the formation of a Δ^3-4-monomethyl intermediate as illustrated in Fig. 2. Lindberg *et al.* suggested that intermediate formation of a 3-ketone would facilitate carbon dioxide loss of the β-keto acid.[21] Recent studies, however, have shown that 3-ketones of 4,4-dimethyl and 4α-methyl triterpenes are not demethylated under non-reducing conditions.[22] It thus seems that the ketones are products rather than early intermediates in the demethylation process. On this basis the mechanism outlined in Fig. 2 represents another possibility. The 4α-methyl group is presumably removed by sequencial oxidation by way of the primary alcohol to the carboxylic

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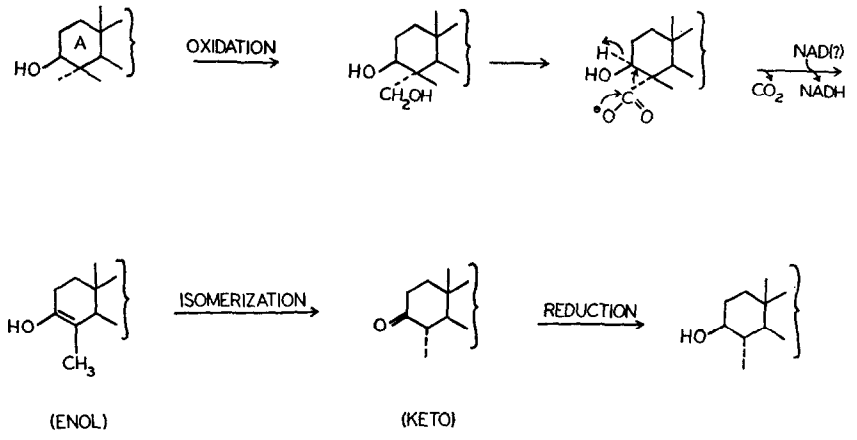

FIG. 2.

acid. Decarboxylation would result in the expulsion of a hydride ion from C-3, compatible with the observed requirement for NAD^+ by the mammalian system.[22] The resulting Δ^3-intermediate is a planar structure and is the enol of the 3-keto tautomer. Formation of the 3-ketone at this stage of the process would explain the involvement of the ketone as a product rather than an early intermediate of the demethylation sequence as has been demonstrated in the mammalian system.[21] In addition, the tautomerization process must be stereospecific with the methyl group assuming the 4α-orientation in the 3-keto-4-monomethyl product. Such a process adequately explains the biosynthesis of 31-norcyclolaudenone. By analogous transformations with reduction of the resulting ketones cycloeucalenol could be formed from 24-methylenecycloartanol and 4α-methyl-8,24-cholestadien-3β-ol from 14-desmethyllanosterol.

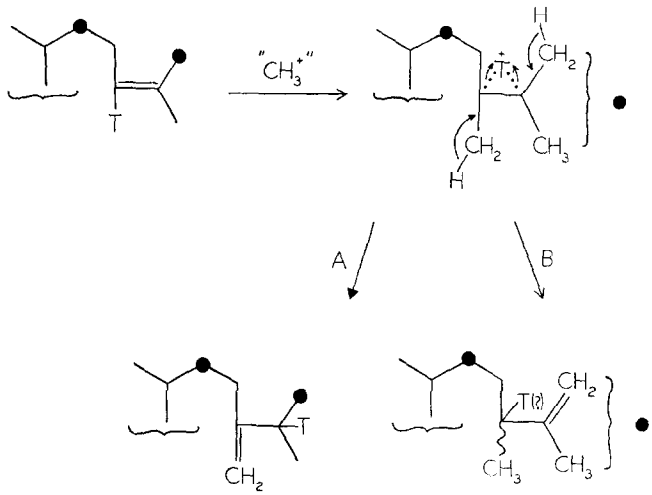

FIG. 3.

These results shed light on another aspect of phytosterol formation. Cyclolaudenol is the analogous 24-methyl-25(26)-en isomer of 24-methylenecycloartanol.[3,4] This is a unique side chain and a mechanism for its formation has been suggested.[23] Since the ^{3}H–^{14}C atomic ratio of 31-norcyclolaudenone is 5:5 and not 4:5 the tritum atom at C-24 must not have been lost during the methylation process. The most likely explanation is that it was retained at C-24 as indicated in Fig. 3B. Electrophilic attack by the methyl donor at C-24 would result in the formation of a bridged carbonium ion. Loss of a proton from the new methyl group at C-24 would then result in the formation of the 24-methylene side chain, with the 'bridged' proton being retained at C-25. Similarly, loss of a C-26 proton with retention of the original C-24 hydrogen at this center would result in the formation of the 24-methyl-25(26)-en side chain. Recent studies have indicated the C-24 hydrogen to be retained during the biosynthesis of cyclolaudenol and 31-norcyclolaudenol in *P. vulgare*.[24]

EXPERIMENTAL

General. All solvents and reagents were analytical grade. The solvents were distilled before use. TLC was performed on 250 μ layers of silica gel G prepared in the usual manner.[1] 31-Norcyclolaudenone had R_f 0·82 in trimethylpentane–EtOAc–HOAc (40:20:0·4). In petrol–benzene (4:1), squalene had R_f 0·86. 2-^{14}C-Mevalonic acid as the dibenzylethylenediamine salt and 3-RS-4R-(4-$^{3}H_1$)-mevalonic acid as the lactone were obtained from Amersham Searle Corp. The (4-$^{3}H_1$)-mevalonic acid was converted to the sodium salt by the action of alkali. These two substrates were mixed to obtain the 3-RS-[2-^{14}C-(4R)-4-$^{3}H_1$] mevalonic acid. The $Ba^{14}CO_3$ and 2-^{14}C-acetic acid were obtained from New England Nuclear. The $^{14}CO_2$ was generated by the action of H_2SO_4 on the carbonate. For samples containing both ^{3}H and ^{14}C a Packard model 3380 liquid scintillation counter was used. The samples were dissolved in 15 ml of scintillator prepared from 6·0 g of 2,5-diphenyloxazole and 0·6 g 1,4-bis-2-(5-phenyl-oxazolyl)-benzene in 1 l. of toluene. The ^{3}H and ^{14}C were counted with efficiencies of 29 and 65%, respectively. For those samples containing only ^{14}C the radioactivity was determined using an Ansitron counter. This instrument counted ^{14}C with an efficiency of 95%.

31-*Norcyclolaudenone.* This triterpene ketone is the C-25(26) unsaturated isomer of cycloeucalenone and has the following physical constants: m.p. 130–132°, $[\alpha]_{27}^{D}$ + 49·0°; infrared bands at 3020 (shoulder, cyclopropane ring), 1698 (>C=O), 1642 and 872 cm^{-1} (=CH_2); NMR vinylic proton absorption at τ5·30 and 5·44 $\left(\begin{matrix}R\\CH_3\end{matrix}\!\!>\!C{=}C\!\!<\!\!\begin{matrix}H\\H\end{matrix}\right)$, and methyl vinyl proton absorption at τ 8·44 $\left(\begin{matrix}R\\CH_2\end{matrix}\!\!>\!C{-}C\!\!<\!\!\begin{matrix}H\\H\\H\end{matrix}\right)$; 70 eV mass spectrum, peaks at *m/e* 424 (M), 409 (M-CH_3), 381, 354 (M-70), 341, 299 (M-side chain) and 300 (M-ring A cleavage); 31-norcyclolaudenol,[25,26] m.p. 135–139°, $[\alpha]_{27}^{D}$ + 40·8°; acetate, m.p. 110°, $[\alpha]_{27}^{D}$ +54·7°; benzoate, m.p. 158°, $[\alpha]_{27}^{D}$ + 61·3°. The stereochemistry of the C/D ring juncture and of the side chain substituents have been determined.[2]

Incubations. The $^{14}CO_2$, 1 day 2-^{14}C-mevalonic acid and 2-^{14}C-acetic acid incubations were performed with 5-g slices of banana peel. The inner surface of the peel was etched with a razor to afford rapid absorption of the substrate. For the long term incubations whole bananas were used. A small area of the outer layer of the peel was removed and the substrate allowed to be absorbed. The piece of tissue was then replaced and covered with transparent tape to prevent drying. After incubation of the 5 g slices, 10 g of carrier banana peel was added and the combined tissue extracted overnight with ethanol. For the long term incubations, 15 g of tissue in the immediate vicinity where the substrate was applied was removed and extracted in the same manner. The ethanolic extracts were evaporated to low volume and then dissolved in ether. The latter was washed successively with 15% KOH and water and then evaporated to dryness. To the lipid obtained by this procedure was added 50 mg of carrier 31-norcyclolaudenone. This combined material was then chromatographed on a column (30 × 1·5 cm) containing 15 g of Merck acid-washed alumina. The column was eluted with petrol (3 fractions, 100 ml each) and then benzene (7 fractions.) The petrol fractions were combined and the labeled squalene purified by preparative TLC. The second through the seventh benzene fractions were shown by TLC to contain the ketone. These were combined and the 31-norcyclolaudenone crystallized from acetone as indicated in Tables 1 and 2.

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