Biosynthesis of Cyclolaudenol in *Polypodium vulgare* Linn

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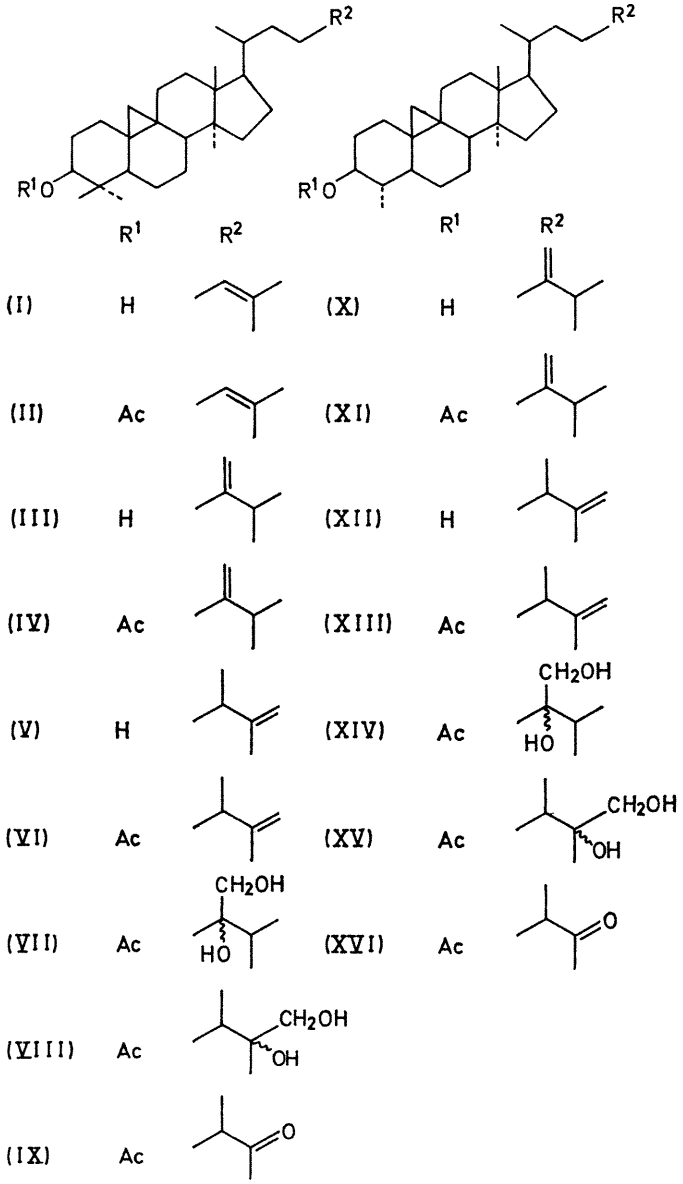
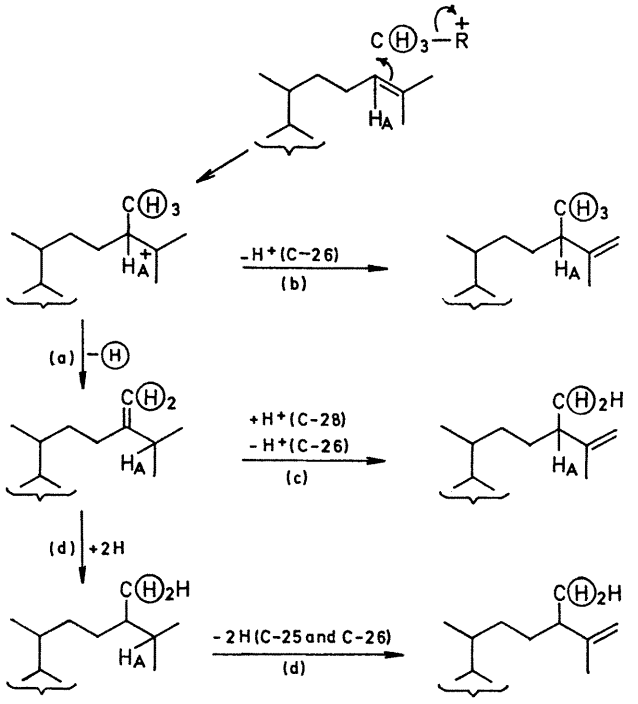
Summary Alkylation of a phytosterol precursor to give cyclolaudenol and 31-norcyclolaudenol involves retention of a hydrogen atom at C-24, elimination of a proton from the terminal methyl group arising from C-3′ of mevalonic acid, and probable retention of all three hydrogen atoms of the incoming methyl group of methionine.

BIOSYNTHESIS of the typical plant sterols requires the



introduction of an alkyl group into the side-chain. The role of *S*-adenosylmethionine as the methyl donor,[1] requirement for a Δ^{24}-sterol precursor,[2] migration of a hydrogen from C-24 to C-25,[3] and formation of an intermediate 24-methylene compound by loss of a proton from the incoming methyl group[4] support the suggested alkylation mechanism[5] [Scheme, route (a)]. However, for triterpenes such as cyclolaudenol (V) with a 25-methylene group a modification of this mechanism is required[6] as indicated by routes (b), (c), and (d) in the Scheme. We now report results of investigations designed to elucidate which mechanism is operative during cyclolaudenol (V) biosynthesis in the rhizomes of *Polypodium vulgare* Linn. The incorporation of [2-^{14}C]mevalonate and [1,24,25,30-$^{3}H_4$]-squalene into fernene, a triterpene hydrocarbon of *P vulgare*, has been reported previously.[7]



SCHEME. *The circled* H *is derived from the methyl group of methionine, whilst* H_A *arises from the* 4-pro-R- *hydrogen of mevalonic acid.*

Examination of the triterpene components of *P. vulgare* rhizomes used in the present work showed the presence of cycloartanol, cyclolaudenol (V), 31-norcycloartanol, and 31-norcyclolaudenol (XII) in agreement with previous reports,[8] but in addition cycloartenol (I), 24-methylenecycloartanol (III), and cycloeucalenol (X) were identified.

It is possible to differentiate between the various mechanisms shown in the Scheme by determining the fate of H_A and the number of hydrogen atoms of the introduced methionine methyl group which are retained in the cyclolaudenol (V). Investigation of the first point was facilitated by the use of 3*R*-[2-^{14}C,(4*R*),4-$^{3}H_1$]mevalonate which in plant tissues will produce cycloartenol (I), the presumed precursor of 24-methylenecycloartanol (III) and cyclolaudenol (V), with ^{14}C at positions 1,7,15,22,26 or 27, and 30 or 31, and tritium in the 3α, 5α, 8β, 17α, 20, and 24 positions.[9]

Rhizome slices (5·0g) and leaves (1·0g) of *P. vulgare* (collected in February 1969) were incubated with 3*R*-[2-^{14}C,(4*R*),$^{3}H_1$]mevalonate (10 μCi ^{14}C) for 24 hr at 25° and the non-saponifiable lipids isolated. After addition of carrier material, chromatography on silica gel gave the squalene, and 4,4-dimethyl-, 4-monomethyl-, and 4-demethyl-triterpene alcohol fractions. The triterpene alcohol fractions were acetylated and subjected to further purification by $AgNO_3$–silica gel t.l.c. (Table). At this stage cyclolaudenyl acetate (VI) was not separated from 24-methylenecycloartanyl acetate (IV), nor was 31-norcyclolaudenyl acetate (XIII) resolved from cycloeucalenyl acetate (XI). Separation of these pairs of compounds was achieved by conversion into the corresponding diols† by treatment with osmium tetroxide–pyridine followed by t.l.c. on silica gel‡ (Table).

Incorporation of 3R-[2-^{14}C,(4R),4-$^{3}H_1$] *mevalonate into* P. vulgare *triterpenes*

Compound	^{3}H : ^{14}C ratio	Normalised ^{3}H : ^{14}C atomic ratio	% Incorp. of MVA
Squalene	10·32	6 : 6	0·35
Cycloartenyl acetate (II)	10·21	5·94 : 6	7·45
Cycloartanyl acetate	10·19	5·92 : 6	0·87
24-Methylenecycloartanyl acetate (IV) plus cyclolaudenyl acetate (VI)	10·36	6·02 : 6	1·75
31-Norcycloartanyl acetate	10·20	4·94 : 5[a]	0·15
1-Norcyclolaudenyl acetate (XIII) plus cycloeucalenyl acetate (XI)	10·63	5·15 : 5[a]	0·85
β-Sitosterol	5·63	2·73 : 5	0·07
24ξ,28-Dihydroxy-24-methylcycloartanyl acetate (VII)	10·26	5·97 : 6	—
25ξ,26-Dihydroxy-24-methylcycloartanyl acetate (VIII)	10·31	5·99 : 6	—
24ξ,28-Dihydroxy-24-methyl-31-norcycloartanyl acetate (XIV)	10·68	5·17 : 5[a]	—
25ξ,26-Dihydroxy-24-methyl-31-norcycloartanyl acetate (XV)	10·37	5·02 : 5[a]	—
25-Oxo-24-methyl-26-norcycloartanyl acetate (IX)	10·66	6·20 : 6	—
25-Oxo-24-methyl-26,31-bisnorcycloartanyl acetate (XVI)	10·32	5·00 : 5[a]	—
(XVI) after base equilibration and re-acetylation	8·05	3·90 : 5	—

[a] Demethylation at C-4 to give the 4-monomethyl-triterpenes involves loss of the 3α-tritium atom and the radioactively labelled 4-methyl group (accompanying communication).

The ^{3}H : ^{14}C ratios of squalene, cycloartenyl acetate (II), and the diols of 24-methylenecycloartanyl acetate (VII) and cyclolaudenyl acetate (VIII) were essentially the same and corresponded to a ^{3}H : ^{14}C atomic ratio of 6 : 6 thus indicating that H_A was retained in all three tetracyclic triterpenes. Similarly the ^{3}H : ^{14}C ratios of the diols of cycloeucalenyl acetate (XIV) and 31-norcyclolaudenyl acetate (XV) were in good agreement and again consistent with the retention of H_A in both compounds. Treatment of diols (VIII) and (XV) with sodium periodate produced the corresponding 25-oxo-compounds, (IX) and (XVI) respectively, with unchanged ^{3}H : ^{14}C ratios. The presence of a tritium atom at C-24 in the 25-methylene-triterpenes was confirmed by equilibration of the 25-oxo-24-methyl-26,31-bisnorcycloartanyl acetate (XVI) with KOH–dioxan under exchange conditions. This resulted in a drop in the ^{3}H : ^{14}C ratio equivalent to the loss of one tritium atom. The conversion of diols (VIII) and (XV) into the corresponding 25-oxo-derivatives (IX) and (XVI), respectively, without change in the ^{3}H : ^{14}C ratios further suggests that the carbon eliminated was not radioactive and therefore not derived from C-2 of mevalonic acid. It is thus probable that the terminal methylene group of cyclolaudenol (V) and 31-norcyclolaudenol (XII) is derived in a stereospecific manner in which a proton is eliminated from the methyl group arising from C-3′ of mevalonic acid, that is the methyl group which is *cis* to the remainder of the cycloartenol side-chain.[10]

The above results eliminated route (d) but did not differentiate between routes (b) and (c). To decide between routes (b) and (c) it was necessary to know the fate of the three hydrogen atoms of the methionine methyl group involved in the alkylation reaction. Deuterium-labelled methionine has been used for this type of study in ergosterol,[4] poriferasterol,[11a] and stigmastenol[11b] biosynthesis. However, the comparatively high endogenous level of cyclolaudenol (V) coupled with the rather poor incorporation of precursors into this compound in *P. vulgare* rhizomes rendered this approach impracticable in the present work. An alternative technique[12] using [*methyl*-^{3}H-^{14}C]methionine had to be employed although interpretation of results obtained by this method is sometimes difficult and open to criticism because of the tritium isotope effect.[13] Incubation of *P. vulgare* rhizomes with dual labelled methionine (approx. ^{3}H : ^{14}C ratio 16·5) gave a radioactive triterpene fraction from which cyclolaudenol (V) and 24-methylenecycloartanol (III) were isolated as their diol derivatives (VIII) and (VII), respectively. The 24-methylenecycloartanol derivative (VII) had a ^{3}H : ^{14}C ratio of 14·1, indicating that about 15% of the tritium originally present in the methionine methyl group was lost. This result is in accord with the operation of a tritium isotope effect of the same order as that previously indicated by the results obtained using this method to study ergosterol biosynthesis in yeast.[12a,13] If route (c) were operative in cyclolaudenol formation it would be expected that 24-methylene cycloartanol (III) and cyclolaudenol (V) from the same plant tissue would have equal ^{3}H : ^{14}C ratios when biosynthesised in the presence of [*methyl*-^{3}H-^{14}C]methionine. However, the cyclolaudenol derivative (VIII) had a ^{3}H : ^{14}C ratio of 17·4, in approximate agreement with the ^{3}H : ^{14}C ratio of the methionine and significantly higher than the 24-methylenecycloartanol derivative (VII). Within the limitations of this type of experiment we therefore believe that these results support route (b) for cyclolaudenol (V) biosynthesis in which the methionine methyl group is transferred to the triterpene side-chain with retention of all three hydrogen

† Compounds were characterised by m.p. and i.r., n.m.r., and mass spectrometry.

‡ The proportion of cyclolaudenol to 24-methylenecycloartanol was approximately 4 : 1, whereas the distribution of radioactivity between these compounds was about 1 : 12. Cycloeucalenol and 31-norcyclolaudenol were present in about equal amounts, but radioactivity was associated principally with the cycloeucalenol.

atoms. The carbonium ion produced is then stabilised by loss of a proton from one of the terminal methyl groups as outlined above.

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