Biosynthesis of Oleanene- and Ursene-Type Triterpenes from [4-^{13}C]Mevalonolactone and [1,2-$^{13}C_2$]Acetate in Tissue Cultures of *Isodon japonicus* Hara[1]

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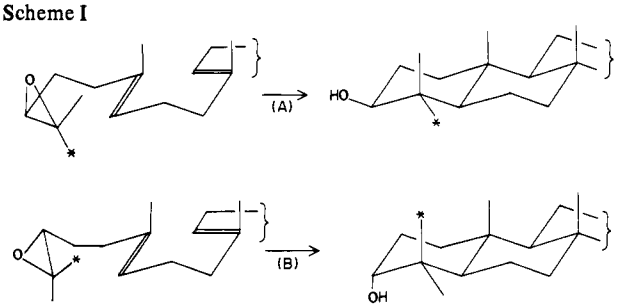
Abstract: Oleanolic, maslinic, 3-epimaslinic, ursolic, and 2α-hydroxyursolic acids were isolated as their methyl esters from tissue cultures derived from *Isodon japonicus* Hara. All ^{13}C NMR signals of the olean-12-enes and the urs-12-enes were assigned by chemical-shift comparisons with those of a number of derivatives and by various NMR techniques. The ^{13}C-labeling patterns were elucidated with the ^{13}C spectra of these triterpenes enriched with [4-^{13}C]mevalonolactone and sodium [1,2-$^{13}C_2$]acetate. During the formation of the D- and E-ring systems, two rearrangements of the carbon skeleton for olean-12-enes and three rearrangements, including methyl migration from C-20 to C-19, for urs-12-enes were verified in the biosynthesis of triterpenes in the tissue cultures. The results were entirely in accord with Ruzicka's hypothesis for cyclization of squalene to β-amyrin and α-amyrin and excluded an alternative mechanism proposed for α-amyrin biosynthesis, including a 19,19-dimethyl intermediate. Moreover, the C-23 and C-30 methyl groups were derived from C-2 of mevalonate and the C-24 and C-29 methyl groups were derived from C-6 of mevalonate in both types of triterpenes. (3*S*)-2,3-Oxidosqualene was confirmed to be a precursor of 3α-hydroxytriterpene, as well as 3β-hydroxytriterpene.

Introduction

A number of triterpenes having olean-12-ene and urs-12-ene skeletons have been isolated from plants. Following the proposal of Woodward and Bloch[3] in 1953, Ruzicka and his co-workers[4] postulated a "biogenetic isoprene rule" for the biosynthesis of terpenoids, including olean-12-ene- and urs-12-ene-type pentacyclic triterpenes, for the mechanism of cyclization from squalene to β-amyrin and α-amyrin. The incorporation of squalene and 2,3-oxidosqualene into β-amyrin is well known.[5] The biogenetic isoprene rule for pentacyclic triterpenes includes 1:2 hydride shifts and two rearrangements of the carbon skeleton for olean-12-enes and three rearrangements for urs-12-enes during the formation of the D- and E-ring systems. Goodwin and co-workers[6] verified the hydride shifts in a biosynthetic study of olean-12-ene by the fact that the tritium atoms derived from (4*R*)-[2-^{14}C,4-^{3}H]-mevalonate (MVA) were distributed at C-3, C-9, C-18, and C-19 (two) in β-amyrin. Barton and co-workers[5a] revealed the same shifts with the aid of 2,3-oxido[11,14-$^{3}H_2$]squalene by the fact that the tritium atoms were distributed at C-11 and C-18 in β-amyrin synthesized in a cell-free preparation of pea seedlings. Arigoni[7] demonstrated that C-23 in sojasapogenol (3β,21β,22β,24-tetrahydroxyolean-12-ene) was derived from C-2 of MVA. However, the rearrangements of the carbon skeleton had not been confirmed experimentally because of the difficulty of studying degradation of oleanene- and ursene-type triterpenes. Also, the hydride shifts had not been verified in the biosynthesis of ursene-type triterpenes.

Since the first incorporation experiment using ^{13}C-labeled precursors in 1970,[8] the application of stable isotopes for tracing metabolism has greatly increased in biosynthetic studies of microbial metabolites,[9] because all labeled sites can be detected and identified easily by ^{13}C FT NMR spectra of the labeled products without chemical degradation. Lower incorporations of ^{13}C-labeled precursors and lower yields of metabolites in higher plants in comparison with those in microorganisms have prevented applications of the ^{13}C NMR method to higher plants.[10] The use of callus cultures of higher plants in biosynthetic studies should have the advantages of higher incorporation and a shorter period of incubation, as well as the fact that the callus can be grown under sterile conditions.[11]

Scheme I



Also, a controversial problem exists concerning the precursor of triterpenoids. (3*S*)-2,3-Oxidosqualene was demonstrated to be the exclusive precursor of β-amyrin in the plant system by Barton and colleagues[5b] (see Scheme IA). On the other hand, Halsall and co-workers[12] and Moss and co-workers[13] proposed an alternative mechanism that the 3α-hydroxytriterpenoid could arise from (3*R*)-2,3-oxidosqualene folded in a boat–chair–chair form (see Scheme IB). Halsall et al.[12] also referred to the possibility of a ketonic intermediate. We successively demonstrated[14] with the aid of radioisotopically labeled precursor in tissue cultures of *Isodon japonicus* Hara and 3α-hydroxytriterpenoid, 3-epimaslinic acid (**6**), is biosynthesized from its 3β isomer, maslinic acid (**5**). The incorporation ratios of (4*R*)-[2-^{14}C,4-^{3}H]MVA incubated with the callus into oleanolic acid (**4**), **5**, and **6** were 1.34, 5.33, and 0.32%, respectively, on the basis of the administered (3*R*)-[2-^{14}C]MVA. [$^{14}C_6$]Maslinic acid prepared biosynthetically from [2-^{14}C]MVA was incorporated into **6** in a ratio of 0.35%. Our results excluded the alternative mechanism involving the cyclization of (3*R*)-2,3-oxidosqualene in the 3α-hydroxytriterpenoid biosynthesis. Thus, the high incorporation ratios of

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(2) Niigata Pharmaceutical College, Niigata, Japan.
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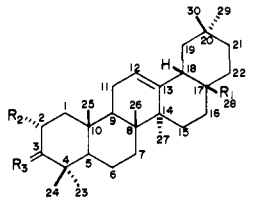
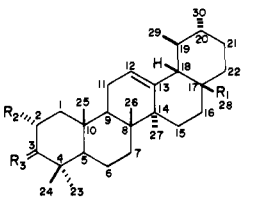
MVA into the triterpenes observed in this study suggested that application of the ^{13}C NMR method could prove Ruzicka's hypothesis as well as confirm the above result.

We report here experimental evidence for the carbon rearrangements in Ruzicka's hypothesis for the cyclization of squalene to the olean-12-ene- and the urs-12-ene-type triterpenes biosynthesized from [4-^{13}C]MVA and [1,2-$^{13}C_2$]acetate in tissue cultures of *I. japonicus*.

Results and Discussion

Isolation and Identification of Sterols and Triterpenes. Callus was derived from stems of *I. japonicus* on Linsmaier and Skoog (L–S) medium containing agar and various concentrations of 2,4-dichlorophenoxyacetic acid (2,4-D) and kinetin and has been subcultured onto fresh medium containing 2,4-D (10^{-6} M) every 3–4 weeks since 1968. Four-week-old tissues were harvested from suspension cultures and extracted with hot methanol. The trimethylsilyl derivative of the sterol fraction was analyzed by gas–liquid chromatography (GLC) and gas chromatography–mass spectrometry (GC–MS) for β-sitosterol (**1**), stigmasterol (**2**), and campesterol (**3**). The acetates of the sterols were separated by silver nitrate impregnated silica gel column chromatography and purified by high-performance liquid chromatography (HPLC) to give β-sitosteryl acetate and stigmasteryl acetate, which were identified by GC–MS and ^{13}C NMR spectroscopy. Methyl oleanolate (**9**) and methyl maslinate (**10**) were isolated by re-





4: R_1 = COOH, R_2 = H, R_3 = α-OH

5: R_1 = COOH, R_2 = OH, R_3 = α-OH

6: R_1 = COOH, R_2 = OH, R_3 = β-OH

9: R_1 = $COOCH_3$, R_2 = H, R_3 = α-OH

10: R_1 = $COOCH_3$, R_2 = OH, R_3 = α-OH

11: R_1 = $COOCH_3$, R_2 = OH, R_3 = β-OH

18: R_1 = CH_3, R_2 = H, R_3 = α-OH

19: R_1 = CH_3, R_2 = H, R_3 = α-OAc

20: R_1 = $COOCH_3$, R_2 = H, R_3 = α-OAc

21: R_1 = $COOCH_3$, R_2 = H, R_3 = O

7: R_1 = COOH, R_2 = H, R_3 = α-OH

8: R_1 = COOH, R_2 = OH, R_3 = α-OH

12: R_1 = $COOCH_3$, R_2 = H, R_3 = α-OH

13: R_1 = $COOCH_3$, R_2 = OH, R_3 = α-OH

14: R_1 = CH_3, R_2 = H, R_3 = α-OH

15: R_1 = CH_3, R_2 = H, R_3 = α-OAc

16: R_1 = $COOCH_3$, R_2 = H, R_3 = α-OAc

17: R_1 = $COOCH_3$, R_2 = H, R_3 = O

crystallization. Compound **10** was compared with a specimen which was synthetically derived from **9** according to Cheung.[15] Methyl 3-epimaslinate (**11**) was isolated and identified by chemical conversion into **10** through a 3-oxo compound. The mother liquors of **9** and **10** were respectively shown by ^{13}C NMR spectra to be a mixture of methyl ursolate (**12**) and **9** and of methyl 2α-hydroxyursolate (**13**) and **10**. They were treated with *m*-chloroperbenzoic acid in methylene chloride to remove **9** and **10** as 12,13-epoxides,[16] giving **12** and **13**, respectively. Diterpenoids which were isolated from the intact plant[17] were not produced in the callus.

^{13}C NMR Signal Assignment. Recently, Doddrell et al.[18] reported ^{13}C NMR spectra of some olean-12-enes and urs-12-enes as the most precise tool for distinguishing between the two types, which is not easy. In a previous paper,[19] we reported full signal assignments of ^{13}C NMR spectra of several olean-12-enes to promote use of this tool for biosynthetic studies as well as for structure determinations. Prior to feeding experiments of ^{13}C-labeled precursor to the callus, we assigned the ^{13}C signals in the complete ^1H-decoupled ^{13}C natural-abundance FT NMR spectra of the methyl esters **9**–**13**. The assignments were made by means of ^1H single-frequency off-resonance decoupling (SFORD) techniques;[20] by application of known chemical-shift rules,[21] such as hydroxy-substitution and acetylation shifts[22] and γ and δ effects;[23] and by comparisons of the spectra among compounds, including those of the olean-12-enes in the previous study.[19] In addition to these techniques, line broadenings of CH_2 and CH resonances of these types of compounds were useful for diagnosis, as described previously.[19] Several ^1H SFORD and selective decoupling experiments[20] were carried out with the ^{13}C spectra of **9** and **12** in $CDCl_3$ containing various amounts of an NMR shift reagent, Eu(fod)$_3$. Since ^1H signals due to the methyl and some other protons in the triterpene–Eu(fod)$_3$ mixtures were assignable as reported previously,[24] the ^{13}C methyl signals were confirmed on the basis of the signal multiplicities and the magnitudes of residual $^{13}C/^1H$ spin couplings. The results shown in Table I confirmed that Knight's assignments[25] for α- and β-amyrins are correct on the basis of similar experiments.

Single ^{13}C Labeling Study Using [4-^{13}C]MVA. Callus cultures of *I. japonicus* have a good ability to incorporate exogenous MVA, as described previously,[14] and this prompted us to feed ^{13}C-labeled MVA to the tissue cultures to confirm the rearrangements of carbon atoms during the formation of olean-12-ene and urs-12-ene skeletons from (3*R*)-2,3-oxidosqualene. Procedures for labeling with radioisotopes and stable isotopes are similar but differ in that larger amounts of a ^{13}C-enriched precursor must be administered. Another report has pointed out[26] that a change in the metabolic pool size resulting from the use of higher precursor concentrations could introduce the risk of metabolic distortion. However, we found that cell growth and the production of triterpenes and sterols in tissue cultures of *I. japonicus* were not affected by a high concentration of MVA (50 mg/300 mL). [4-^{13}C]MVA (**22**) (about 30 atom % labeled at C-4) prepared from sodium [2-^{13}C]acetate by Conforth's method[27] was fed to 2-week-old callus of *I. Japonicus* grown on L–S liquid medium. After 2 more weeks of incubation, the enriched metabolites **36**, **40**, and **42** were isolated as their methyl esters, **37**, **41**, and **43**, respectively. The complete ^1H-decoupled ^{13}C NMR spectra of the methyl esters were compared between the enriched and the unenriched samples in $CDCl_3$. The spectrum of methyl [$^{13}C_6$]3-epimaslinate (**37**) biosynthesized from [4-^{13}C]MVA clearly showed that the six carbons, C-3, C-5, C-9, C-13, C-18, and C-19, were enriched about twofold (Table I). The results were entirely in accord with Ruzicka's hypothesis[4] for cyclization from squalene to β-amyrin, **27** → **28** → **29** → **30** → **31**, as shown in Scheme II. Similar ^{13}C-labeling patterns were observed in the spectra of **33** and **35**, which were conveniently determined as mixtures of the corresponding ursenes **41** and **43**, respectively; **33** and **35** were enriched about five- and twofold, respectively.

In the biosynthesis of ursene-type compounds **40** and **42**, the D ring was also formed via the process **27** → **28**, as demonstrated for the biosynthesis of the oleanene-type triterpenes, because about

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Table I. Carbon-13 Chemical Shifts (δ_C) of Urs-12-enes and Olean-12-enes

carbon no.	14	18	15	19	12 (41)[a]	9 (33)[a]	16	20	17	21	13 (43)[a]	10 (35)[a]	11 (37)[a]
C-1	38.7	38.5	38.4	38.2	38.8	38.5	38.3	38.1	39.3	39.1	46.8	46.4	41.7
C-2	27.2	27.0	23.6	23.6	27.3	27.1	23.6	23.6	33.9	34.2	68.9	68.8	66.5
C-3	78.8	78.9	80.7	80.7	*78.8*	*78.7*	80.7	80.7	216.5	216.8	*83.8*	*83.8*	*78.9*
C-4	38.7	38.7	37.6	37.6	38.8	38.7	37.6	37.5	47.1	47.3	39.1	39.1	38.5[b]
C-5	55.2	55.1	55.3	55.3	*55.4*	*55.2*	55.3	55.2	55.2	55.2	*55.4*	*55.3*	*48.1*
C-6	18.3	18.3	18.3	18.3	18.4	18.3	18.1	18.2	19.5	19.5	18.4	18.3	18.1
C-7	32.9	32.6	32.8	32.6	33.0	32.6	32.8	32.6	32.4	32.1	32.9	32.6	32.5
C-8	40.0	39.7	40.1	39.7	39.6	39.3	39.5	39.3	39.1	39.1	39.6	39.1	39.7[b]
C-9	47.7	47.6	47.6	47.6	*47.5*	*47.6*	47.4	47.5	46.6	46.7	*47.5*	*47.5*	*47.4*
C-10	36.9	37.0	36.8	36.8	37.0	37.0	36.8	36.9	36.6	36.6	38.3	38.3	38.3[b]
C-11	23.3	23.4	23.2	23.4	23.3	23.4	23.2	23.6	23.4	23.5	23.4	23.5	23.4
C-12	124.3	121.7	124.1	121.5	125.5	122.1	125.4	122.1	125.0	121.9	125.3	122.0	122.1
C-13	129.3	145.0	139.4	144.9	*138.0*	*143.4*	138.0	143.6	137.9	143.5	*138.1*	*143.6*	*143.8*
C-14	42.0	41.7	42.1	41.7	42.0	41.6	41.9	41.6	41.9	41.7	42.1	41.7	41.9
C-15	28.7	26.2	28.7	26.2	28.2	27.7	28.1	27.7	27.9	27.6	28.0	27.6	27.7
C-16	26.6	27.3	26.7	27.0	24.3	23.1	24.2	23.0	24.1	23.0	24.3	23.1	23.2
C-17	33.7	32.5	33.8	32.5	48.1	26.6	48.0	46.6	47.9	46.7	48.1	46.6	46.8
C-18	58.9	47.2	59.0	47.2	*52.8*	*41.3*	52.8	41.3	52.8	41.2	*52.8*	*41.3*	*41.3*
C-19	39.6[b]	46.8	39.7[b]	46.8	*39.1*	*45.8*	38.9	45.8	38.8	45.7	*39.1*	*45.8*	*46.0*
C-20	39.6[b]	31.1	39.7[b]	31.1	38.8	30.6	38.9	30.6	38.8	30.5	38.9	30.7	30.7
C-21	31.2	34.8	31.3	34.8	30.7	33.8	30.7	33.8	30.5	33.7	30.7	33.8	34.0
C-22	41.5	37.2	41.5[b]	37.1	36.7	32.3	36.6	32.3	36.6	32.1	36.7	32.3	32.5
C-23	28.1	28.1	28.1	28.1	28.2	28.1	28.1	28.0	26.5	26.4	28.7	28.6	28.5
C-24	15.6	15.5	16.8	16.8	15.5[b]	15.6[b]	16.9	16.8	21.3[b]	21.3	17.0	16.8	21.9
C-25	15.6	15.5	15.7	15.7	15.7[b]	15.3[b]	15.5	15.3	15.1	14.8	17.0	16.8	16.4
C-26	16.8	16.8	16.8	16.8	16.9	16.8	16.9	16.8	16.8	16.7	17.0	16.8	17.0
C-27	23.3	26.0	23.2	26.0	23.6	26.0	23.6	25.8	23.4	25.7	23.7	26.0	26.2
C-28	28.1	28.3	28.1	28.3	177.7	177.9	177.6	177.8	177.3	177.7	177.9	178.0	178.1
C-29	17.4	33.2	17.5	33.4	16.9	33.1	17.1	33.1	16.8	33.0	17.0	33.1	33.2
C-30	21.3	23.6	21.4	23.6	21.2	23.6	21.2	23.6	21.1[b]	23.5	21.2	23.5	23.6
CO_2Me					51.4	51.3	51.3	51.4	51.2	51.3	51.5	51.5	51.5
OCO*Me*			21.2	21.2			21.1	21.2					
OCOCMe			170.4	170.4			170.5	170.5					

[a] Figures italicized are the ^{13}C chemical shifts for the compounds in parentheses, which are biosynthesized from [4-^{13}C]MVA (Scheme II). [b] Assignments may be reversed in each vertical column.

five- and twofold ^{13}C enrichment was clearly observed at C-18 in the spectra of **41** and **43**, respectively, together with that at C-3, C-5, C-9, and C-13 (Table I). Two possible mechanisms for the E-ring formation have been considered thus far.[4,28,29] If cation **44** or its equivalent is an intermediate for α-amyrin, one of the enriched carbons originating from [4-^{13}C]MVA should be situated at C-20. On the other hand, if the E ring is formed via the process **28** → **29** → **30** → **38** → **39**, the enriched carbon due to [4-^{13}C]MVA should be at C-19. Although one signal corresponding to C-19 in **41** and **43** was enriched about five- and twofold, respectively, the assignment of the enriched signal remained ambiguous because the C-19 and C-20 signals in these compounds were positioned close to each other.

If doubly labeled [1,2-$^{13}C_2$]acetate is incorporated into ursene-type triterpenes, we can distinguish between these two possible mechanisms from the labeling patterns as shown in Scheme II.

Double ^{13}C Labeling Study Using [1,2-$^{13}C_2$]Acetate. If the process **47** → **63** → **64** operates for the formation of the E-ring system in ursene-type triterpenes, C-20 and C-21 should be derived from the same acetate molecule and the C-21 signal should appear as a doublet coupled to C-20. On the other hand, the C-21 signal should be a singlet due to a singly labeled carbon, if the alternative process **47** → **48** → **49** → **50** → **51** operates. Furthermore, the labeling patterns of the methyl groups C-23, C-24, C-29, and C-30 in the ursenes and the oleanenes should be distinguishable, since these methyls are derived from C-2 and from C-6 of MVA, respectively. A mixture of sodium [1,2-$^{13}C_2$]acetate and unlabeled sodium acetate (1:2) was fed to 4-week-old callus. After 2 more weeks of incubation, enriched ursenes **52** and **54** were isolated as their methyl esters **53** and **55**. The complete ^1H-decoupled ^{13}C NMR spectra of **53** and **55**, overlapping with the natural-abundance ^{13}C spectra, clearly showed 18 doublets and 12 singlets (see Table II). The signals of C-1, C-7, C-15, and C-22 were singlets

Scheme II

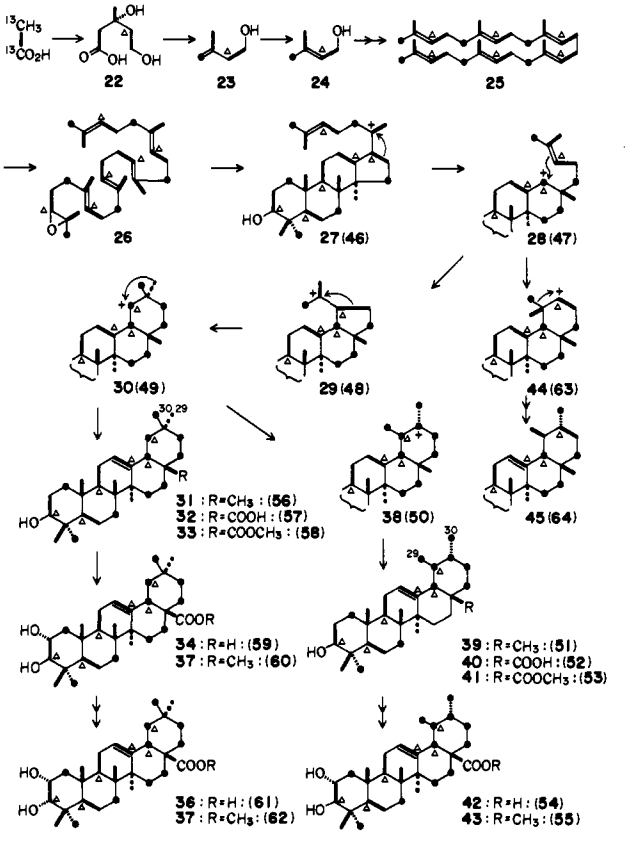

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Table II. Carbon-13 Spectral Data of Methyl Ursolate (53), 2α-Hydroxyursolate (55), and 3-Epimaslinate (62) from [1,2-$^{13}C_2$]Acetate

	53			55			62		
carbon no.	δ_C	multiplicity	J, Hz	δ_C	multiplicity	J, Hz	δ_C	multiplicity	J, Hz
C-1	38.8	s		46.8	s		41.7	s	
C-2	27.3	d	38[a]	68.9	d	38	66.5	d	39
C-3	78.8	d	36	83.8	d	38	78.9	d	37
C-4	38.8	d	38[a]	39.1	d	36[a]	38.5[b]	d	35[a]
C-5	55.4	d	35	55.4	d	36	48.1	d	34
C-6	18.4	d	36	18.4	d	36[a]	18.1	d	35[a]
C-7	33.0	s		32.9	s		32.5	s	
C-8	39.6	d	38[a]	39.6	d	38[a]	39.7[b]	d	36[a]
C-9	47.5	d	36[a]	47.5	d	36[a]	47.4	d	34[a]
C-10	37.0	d	38[a]	38.3	d	37[a]	38.3[b]	d	35[a]
C-11	23.3	d	36[a]	23.4	d	36[a]	23.4	d	35
C-12	125.5	d	71	125.3	d	73	122.1	d	73
C-13	138.0	d	72	138.1	d	73	143.8	d	72
C-14	42.0	d	37[a]	42.1	d	36[a]	41.9	d	36[a]
C-15	28.2	s		28.0	s		27.7	s	
C-16	24.3	s		24.3	s		23.2	s	
C-17	48.1	d	56[a]	48.1	d	56[a]	46.8	d	55[a]
C-18	52.8	s		52.8	s		41.3	s	
C-19	39.1	s		39.1	s		46.0	s	
C-20	38.8	s		38.9	s		30.7	d	36
C-21	30.7	s		30.7	s		34.0	s	
C-22	36.7	s		36.7	s		32.5	s	
C-23	28.2	s		28.7	s		28.5	s	
C-24	15.5[b]	d	38[a]	17.0	d	38[a]	21.9	d	36[a]
C-25	15.7[b]	d	38[a]	17.0	d	38[a]	16.4	d	36
C-26	16.9	d	37[a]	17.0	d	37[a]	17.0	d	36[a]
C-27	23.6	d	36	23.7	d	36	26.2	d	35
C-28	177.7	d	56	177.9	d	56	178.1	d	55
C-29	16.9	s		17.0	s		33.2	d	36[a]
C-30	21.2	s		21.2	s		23.6	s	

[a] These J values were accurate to ±2 Hz because of overlapping of one peak of the doublet signals with other signals. [b] Assignments may be reversed.

as expected, being those derived from C-2 of MVA. The fact that C-16 and C-18 appeared as singlets agreed with the process **46** → **47** for the formation of the D-ring system, as demonstrated in the single labeling study.

The fact that the signal due to C-21 appeared as a singlet like that due to C-19 indicated that the process **47** → **48** → **49** took place and the process **47** → **63** did not. Consequently, an alternative mechanism including the 19,19-dimethyl intermediate **63** could be excluded.

Furthermore, the C-30 and C-20 signals in **53** and **55** were also singlets like that of C-29. This strongly suggested that the C-30 methyl group originated from the C-20β methyl group in the intermediate **49** and, hence, was derived from C-2 of MVA. The C-29 methyl group probably turned out to have originated from the C-20α methyl group in **49** and was derived from C-6 of MVA. The C-23 singlet and the C-24 doublet were derived from C-2 and C-6 of MVA, respectively.

These ^{13}C double-labeling patterns confirm that the biosynthesis of ursene-type triterpenes proceeds via the route **46** → **47** → **48** → **49** → **50** → **51** postulated earlier.[4]

The ^{13}C spectra of oleanene **62** obtained simultaneously from [1,2-$^{13}C_2$]acetate exhibited 20 doublets and 10 singlets, as shown in Table II. The signals due to C-1, C-7, C-15, and C-22 appeared as singlets as expected, being derived from C-2 of MVA. The biosynthetic process **46** → **47** was represented by the fact that the C-16 and C-18 signals appeared as singlets, and the singlet signals due to C-19 and C-22 accounted for the process **47** → **48** → **49**. These ^{13}C-labeling patterns also confirmed the biosynthetic mechanism of oleanene-type triterpenes (**46** → **47** → **48** → **49** → **56**) and showed that both oleanene- and ursene-type triterpenes are formed via the process involving the same intermediate, **49**.

The C-24 and C-29 methyl signals of **62**, each appearing as a doublet, and the C-23 and C-30 methyl signals, each appearing as a singlet, demonstrated that the former and the latter two carbons were derived from C-6 and C-2 of MVA, respectively; this was also confirmed by the fact that the C-20 and C-29 signals in **62** appeared as an AB-type quartet at δ 30.7 and 33.2 (J = 36 Hz), which agreed with the results of earlier studies by Arigoni[7] and Suga et al.[30]

Furthermore, the finding that the C-23 methyl in **62** having a 3α-hydroxy group was derived from C-2 of MVA as well as that of 3β-hydroxytriterpenes **53** and **55** confirmed our result reported earlier;[14] i.e., (3*S*)-2,3-oxidosqualene is a precursor of 3α-hydroxytriterpene (Scheme I).

Conclusion

The ^{13}C-labeling patterns from [4-^{13}C]MVA and sodium [1,2-$^{13}C_2$]acetate were elucidated by the ^{13}C NMR spectra of methyl esters of oleanolic acid (**4**), maslinic acid (**5**), 3-epimaslinic acid (**6**), ursolic acid (**7**), and 2α-hydroxyursolic acid (**8**) isolated from tissue cultures derived from *I. japonicus*. The biogenetic isoprene rule proposed by Ruzicka et al. for cyclization of squalene to β-amyrin and α-amyrin was verified experimentally, since carbon rearrangements occurred during the D- and E-ring system formation in the biosynthesis of olean-12-enes and urs-12-enes and the C-29 methyl group in urs-12-enes was derived from C-6 of MVA. The present results excluded alternative mechanisms proposed for the α-amyrin biosynthesis, including the 19,19-dimethyl intermediate, and for the 3α-hydroxytriterpene biosynthesis, including (3*R*)-2,3-oxidosqualene.

Experimental Section

Melting points were taken on a Kofler hot-stage apparatus and are uncorrected. Sodium [2-^{13}C]- and [1,2-$^{13}C_2$]acetate were purchased from M.S.D. (Canada). ^{13}C FT NMR spectra were recorded on a Varian NV-14 spectrometer operating at 15.087 MHz in [^2H]chloroform using 8-mm spinning tubes at ordinary probe temperature (30 °C). FT NMR

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measurement conditions were as follows: spectral width, 3923 Hz; pulse flipping angle, 16–22° (10–15 μs); acquisition time, 0.6 or 1.0 s; number of data points, 4820 or 7846. Chemical shifts were expressed by δ [in parts per million downfield from internal $(CH_3)_4Si$]. Accuracies of chemical shifts and J values were about ±0.1 ppm and ±1 Hz, respectively. ^1H NMR spectra were taken on a Varian A-56/60D spectrometer using $(CH_3)_4Si$ as an internal standard with accuracies of ±0.02 ppm. Mass spectra (MS) were determined on a Hitachi RMU-6 mass spectrometer. Thin-layer chromatography (TLC) was carried out on glass plates precoated with silica gel GF_{254} (Merck). Workup means washing of extracts with water, drying with Na_2SO_4, filtration, and evaporation under reduced pressure. Gas–liquid chromatography (GLC) was run on a Shimazu Gas Chromatograph GC-4A (RF) fitted with a flame-ionization detector, using a glass U column 1.5 m × 4 mm o.d. packed with 5% SE-30 on Gas Chrom Q (100/120).

Tissue Culture and Preparative Media. The stems of *I. japonicus* were sterilized in 70% alcohol, immersed in 0.2% aqueous mercuric chloride for 20 min, and then washed three times with sterilized water. The stems were cut to an appropriate size (0.7–1.0 cm). The explants were placed under the sterile condition of L–S medium containing 0.8% agar, 2,4-dichlorophenoxyacetic acid (2,4-D, 0–10^{-4} M), and kinetin (0–10.0 ppm). After 3–4 weeks, white callus which had formed from these explants was inoculated onto medium containing 10^{-6} M 2,4-D and 0.2 ppm of kinetin. This callus was subcultured on L–S medium supplemented with 10^{-6} M 2,4-D every 4 weeks in the dark at 25 °C.

Extraction of Triterpenes and Sterols from *I. japonicus* Tissue Cultures. Callus derived from stems of *I. japonicus* was incubated on L–S liquid medium (7.8 L) containing 10^{-6} M 2,4-D, distributed in 26 500-mL conical flasks on a rotary shaker (100 rpm) in the dark at 25 °C. After 4 weeks of incubation, the cultured cells (wet wt 1620 g) were collected and extracted with hot methanol (2 L × 3). The solvent was removed to obtain the residue (11.37 g). The butan-1-ol soluble part of the residue was suspended in an ether–methanol solution and methylated with an excess of diazomethane. After the solvent was evaporated, the methylated mixture was separated with a silica gel lobar column (Merck, size B) eluted with hexane–ethyl acetate–chloroform–acetonitrile (4:1:1:0.5–1) into six fractions: fr-1 (590 mg), -2 (455 mg), -3 (250 mg), -4 (180 mg), -5 (1080 mg), and -6 (664 mg).

Isolation of β-Sitosterol (1) and Stigmasterol (2). Fr-3 was crystallized from methanol to afford colorless crystals, its trimethylsilyl ether was analyzed for **1**, **2**, and campesterol (3) by GLC and GC–MS, and its acetate was separated into a β-sitosteryl acetate fraction (150 mg) and a stigmasteryl acetate fraction (70 mg) by 30% silver nitrate impregnated silica gel column chromatography (hexane–chloroform, 4:1). β-Sitosteryl acetate was purified by HPLC using a Spherisorb ODS 5 μm, 10 × 250 mm column (solvent: acetonitrile–water, 20:1).

Isolation of Methyl Oleanolate (9) and Methyl Ursolate (12). Fr-2 was recrystallized from methanol to give **9** (51 mg), mp 205–206 °C, $[\alpha]^{23.5}_D$ +75.3 ($CHCl_3$), which was identified with an authentic specimen by ^1H and ^{13}C NMR, IR, MS, and mixture melting point. The mother liquor was evaporated, the residue (40.5 mg) was dissolved in methylene chloride and mixed with sodium bicarbonate (300 mg) and *m*-chloroperbenzoic acid (240 mg) in methylene chloride (5 mL), and the solution was stirred at room temperature. After 4 h, the reaction mixture was extracted with methylene chloride and worked up. The residue was chromatographed on silica gel (hexane–ethyl acetate–chloroform–acetonitrile) and gave **12** (20 mg), mp 115–116 °C, $[\alpha]^{25.0}_D$ +67.0 ($CHCl_3$), which was identical with an authentic specimen kindly supplied by Dr. Takeuchi of this Company, and methyl 11,12-epoxyoleanolate, mp 210–213 °C, $[\alpha]^{24}_D$ + 20.9 ($CHCl_3$).

Isolation of Methyl Maslinate (10) and Methyl 2α-Hydroxyursolate (13). Fr-5 was recrystallized from acetone repeatedly to give **10** (45 mg): mp 232–234 °C; $[\alpha]^{23.5}_D$ +59.1 ($CHCl_3$); ^1H NMR ($CDCl_3$) δ_H 0.73, 0.82, 0.90, 0.93, 0.98, 1.03, 1.13 (each 3 H, s), 3.0 (1 H, d, J = 9 Hz, H-3), 3.60 (1 H, m, H-2), 3.62 (3 H, s, OCH_3), 5.29 (1 H, br, $W_{1/2}$ = 6 Hz). Found: C, 76.57; H, 10.24. $C_{31}H_{50}O_4$ requires C, 76.50; H, 10.36. Methyloleanone (100 mg) prepared from Jones' oxidation of **9**, was dissolved in dry benzene and then lead tetraacetate (70 mg) and boron trifluoride–ether (0.1 mL) were added. after the mixture stirred in the dark for 3 h at room temperature, water was added and then the reaction mixture was extracted with ether and worked up to give a raw α-acetoxy ketone (80 mg). The mixture of the raw acetoxy ketone (80 mg) in dioxane (5 mL) and sodium borohydride (100 mg) in an ammonia–ammonium chloride buffer at pH 8.0 (2.5 mL) was stirred under ice cooling for 30 min. Extraction with ether after the addition of water, workup, and crystallization from ether–petroleum ether gave needles (50 mg): mp 194–195 °C; IR ($CHCl_3$) 1730, 1260, 1200 cm^{-1}. The needles were treated with 3% potassium hydroxide in methanol and then worked up, and the residue was recrystallized from acetone to give **10** (27 mg), identified with the specimen isolated from the callus of *I. japonicus* by ^1H and ^{13}C NMR, IR, mixture melting point, and optical rotation. The mixture of mother liquor (1.08 g) containing **10** and **13**, *m*-chloroperbenzoic acid (780 mg), and sodium bicarbonate (800 mg) in methylene chloride (10 mL) was stirred for 5 h at room temperature. The reaction mixture was extracted and worked up. The residue was chromatographed on a silica gel lobar column (Merck, size B) (hexane–ethyl acetate–chloroform–acetonitrile, 2:1:1:1) to give **13** (386 mg) together with methyl 12,13-epoxymaslinate (320 mg). **13**: mp 211–214 °C; $[\alpha]^{25}_D$ +53.0 ($CHCl_3$); ^1H NMR ($CDCl_3$) δ_H 0.75, 0.82, 0.98, 1.02, 1.08 (each 3 H, s), 2.97 (1 H, d, J = 9 Hz, H-3), 3.48 (1 H, m, H-2), 3.58 (3 H, s, OCH_3), 5.25 (1 H, $W_{1/2}$ = 6 Hz, H-12). Found: C, 76.76; H, 10.07. $C_{31}H_{50}O_4$ requires C, 76.50; H, 10.36.

Isolation of Methyl 3-Epimaslinate (11). Fr-4 was crystallized from acetone to give **11** (150 mg) as colorless needles: mp >300 °C; $[\alpha]^{24}_D$ +61.6 ($CHCl_3$); ^1H NMR ($CDCl_3$) δ_H 0.72, 0.86, 0.90, 0.92, 0.96, 1.02, 1.14 (each 3 H, s), 3.41 (1 H, d, J = 3 Hz, H-3), 3.62 (3 H, s, OCH_3), 4.0 (1 H, m, $W_{1/2}$ = 20 Hz, H-2), 5.28 (1 H, $W_{1/2}$ = 7 Hz, H-12). Found: C, 76.84; H, 10.07. $C_{31}H_{50}O_4$ requires C, 76.50; H, 10.36. The mixture of **11** (80 mg), *p*-nitrobenzoyl chloride (64 mg), and pyridine (1 mL) in ether (10 mL) was stirred for 2 h at room temperature and worked up as usual. The residue was purified by silica gel TLC (chloroform–acetone, 9:1). The 2-*O*-*p*-nitrobenzoate of **11** (55 mg) was obtained: ^1H NMR ($CDCl_3$) δ_H 0.74 (3 H, s, CH_3), 0.92 (6 H, s, CH_3 × 2), 0.98, 1.05, 1.08, 1.15 (each 3 H, s, CH_3), 3.62 (3 H, s, OCH_3), 3.65 (1 H, d, J = 3 Hz, H-3), 5.02–5.67 (2 H, br, H-12 and H-2), 8.23 (4 H, ArH). The 2-*O*-*p*-nitrobenzoate (50 mg) was oxidized with Jones' reagent in acetone at 10 °C for 10 min and worked up as usual. The residue was purified by silica gel TLC (chloroform–acetone, 20:1) to give a 2-*O*-*p*-nitrobenzoyl-3-oxo compound (50 mg). The 3-oxo compound (45 mg) was reduced with $NaBH_4$ in ethanol at room temperature for 1 h and worked up as usual. The residue was purified by silica gel TLC (benzene–acetone, 9:2) to give **19** (33 mg), which was identical with the sample isolated from the callus of *I. japonicus*.

Effects of MVA on Sterol and Triterpene Metabolism. The callus of *I. japonicus* was incubated on L–S liquid medium containing 2,4-D (10^{-6} M) and MVA (0, 50, and 350 mg/300 mL) on a rotary shaker (100 rpm) in the dark at 25 °C. After 4 weeks of incubation, the cultured cells in the flasks were collected and extracted with hot methanol (300 mL × 2) and chloroform (300 mL × 1). The solvents were combined and evaporated under reduced pressure. The residue was partitioned with butan-1-ol and water. The organic layer was washed with water and evaporated to give a residue, which was suspended in an ether–methanol solution. An excess of diazomethane was added to this solution, which was then left standing at room temperature overnight. After the solvent was evaporated, the residue was separated with a silica gel lobar column (Merck, size B), eluted with hexane–ethyl acetate–chloroform–acetonitrile (4:1:1:0–1), into four fractions: sterol, **9** and **12**, **10** and **13**, and **11**. The results obtained are shown in Table III.

Table III. Effects of MVA on Sterol and Triterpene Metabolism in Tissue Cultures of *I. japonicus*

	amount of MVA, mg/300 mL		
	0	50	350
wet wt	68.9 g	70.2 g	48.5 g
dry wt	3.0 g	3.2 g	2.5 g
sterols	14.0 mg	14.7 mg	31.8 mg
methyl oleanolate (**9**) methyl ursolate (**12**)	10.0 mg	7.3 mg	11.7 mg
methyl maslinate (**10**) methyl 2α-hydroxyursolate (**13**)	20.7 mg	14.4 mg	25.5 mg
methyl 3-epimaslinate (**11**)	7.4 mg	7.3 mg	10.0 mg

[4-^{13}C]Mevalonolactone. This was synthesized according to Conforth's procedure from 4,4-dimethoxybutan-2-one and ethyl [2-^{13}C]bromoacetate, which contained 30 atom % of ^{13}C at C-2.

Incubation of [4-^{13}C]Mevalonolactone. [4-^{13}C]Mevalonolactone (1 g) dissolved in 50% aqueous ethanol (7.2 mL) was added to 2-week-old callus of *I. japonicus* grown on L–S liquid medium (4.8 L) supplemented with 10^{-7} M 2,4-D, distributed in 16 500-mL conical flasks, and the incubation was continued for 2 more weeks at 25 °C with rotary shaking. The cells (dry wt 24.1 g) were harvested and extracted with hot methanol, β-sitosteryl acetate (31 mg), **41** (16 mg), **43** (29 mg), and **37** (30 mg), together with methyl 12,13-epoxyoleanolate (15 mg) and methyl 12,13-epoxymaslinate (43.6 mg), and then were isolated as described above.

Incubation of Sodium [1,2-$^{13}C_2$]Acetate. Two-week-old cultured cells

of *I. japonicus* were inoculated into 20 500-mL conical flasks, each containing 100 mL of L-S liquid medium with 10^{-7} M 2,4-D. Sodium [1,2-$^{13}C_2$]acetate (300 mg) and unlabeled sodium acetate (600 mg) in the same sterilized medium (4 L) were added equally to the callus grown for 4 weeks after inoculation. After 2 more weeks of incubation, the cultured cells (wet wt 600 g) were harvested and extracted with hot methanol. β-Sitosteryl acetate (99 mg), **53** (43 mg), **55** (54 mg), and **62** (30 mg) were isolated as described above, together with methyl 12,13-epoxyoleanolate (55 mg) and methyl 12,13-epoxymaslinate (60 mg).

Sequential Polypeptides of Elastin: Cyclic Conformational Correlates of the Linear Polypentapeptide

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Abstract: Cyclic oligomers of the repeating pentamer sequence of the elastic fiber, $(Val_1\text{-}Pro_2\text{-}Gly_3\text{-}Val_4\text{-}Gly_5)_n$, were synthesized with n = 1–6, and the cyclic oligomers were studied by means of proton and carbon-13 nuclear magnetic resonance, using methods which delineate polypeptide secondary structure. For each of the six cyclic peptides, the temperature dependences of peptide N*H* chemical shifts were determined in water (0 to 90 °C) and Me_2SO (20 to 90 °C), the solvent dependences of peptide N*H* chemical shifts were reported for a $Me_2SO \rightarrow H_2O$ solvent titration, and the solvent dependences of the peptide *C*–O chemical shifts were determined for a $Me_2SO \rightarrow D_2O$ solvent titration. These results were compared to those of the linear polypentapeptide in order to determine which cyclic structure would have a conformation most closely related to the conformation of the linear polymer. The conformations of the cyclopentapeptide and of the cyclodecapeptide were clearly different from that of the linear polypentapeptide, whereas those for n = 3–6 were quite similar. In particular, the cyclopentadecapeptide (n = 3) and the cyclotricosapeptide (n = 6) were found to be excellent cyclic conformational correlates of the linear high polymer. These results were discussed relative to the pitch, number of residues per turn, and helix sense of the β-spiral of the linear polypentapeptide.

Tropoelastin, the soluble precursor protein of fibrous elastin,[1,2] has been shown by Gray, Sandberg, and their colleagues[3,4] to contain the related sequential polypeptides $(Val_1\text{-}Pro_2\text{-}Gly_3\text{-}Gly_4)_n$, $(Val_1\text{-}Pro_2\text{-}Gly_3\text{-}Val_4\text{-}Gly_5)_n$, and $(Ala_1\text{-}Pro_2\text{-}Gly_3\text{-}Val_4\text{-}Gly_5\text{-}Val_6)_n$. This laboratory has synthesized monomers, oligomers, and high polymers of these repeat sequences, has derived secondary structures for these repeats by using proton and carbon-13 nuclear magnetic resonance, and has proposed β-spiral working models for the helical generation of the repeating conformational units.[5]

In developing structures for a synthetic, voltage-dependent transmembrane channel, a concept of cyclic conformations with linear conformational correlates was derived.[6] The concept states that, if there is a describable and relatively strain-free cyclic structure comprised of a substantial number of residues (preferably of a small number of repeat sequences), the process of breaking a single backbone bond and making only minor changes in torsion angles can convert the cyclic structure to a linear helical structure with approximately the number of residues in the cyclic structure becoming the number of residues per turn of helix.[6] While this allows one to conceive of linear structures based on described cyclic structures, it can also be used experimentally in an inverse manner to determine if there are cyclic structures of repeat sequences which conformationally appear nealy identical with a linear sequential polypeptide of interest. This approach is used in the present and future studies from our laboratory.

The linear sequential polypeptides of elastin are each seen experimentally on the nuclear magnetic resonance (NMR) time scale as being comprised of conformationally equivalent repeats.[5] Accordingly, the approach is to study cyclic peptides with increasing numbers of repeating units, to look for those cyclic structures which maintain their symmetry in solution on the NMR time scale, and then, specifically, to search out that cyclic structure whose secondary structure is most like that of the linear sequential polypeptide. When a symmetric cyclic structure is identified with a secondary structure that is essentially indistinguishable from that of the linear structure, it will be analyzed in detail in terms of NMR-derived torsion angles and conformational energy calculations, taking advantage of the symmetry, to achieve a complete description. The cyclic constraint can then be removed and local energy minima can be explored as starting points for generating a linear helical structure, i.e., the linear conformational correlate. The advantages of utilizing cyclic structures in this way are the simplifying symmetry, the presence of fine structure in the NMR from which torsion angles may be obtained, and the added possibility that they may form crystals suitable for X-ray diffraction studies which could provide a check on the solution and conformational energy results.

The objective of the present report is to identify those cyclic peptides, comprised of oligomers of the repeat pentapeptide, which appear to have a conformation similar to that of the linear polypentapeptide. Specifically, the methods to delineate secondary structure will be the temperature dependence of peptide N*H* chemical shifts in water and dimethyl sulfoxide, the solvent dependence of peptide N*H* shifts, and the solvent dependence of peptide *C*–O chemical shifts using dimethyl sulfoxide and water as the solvent pair. The resulting patterns for temperature and solvent dependences of chemical shift will be used as fingerprints for comparing the conformations of cyclic and linear molecules.

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