Stereospecificity in the Side-chain Formation of 24β-Ethylsterols in Tissue Cultures of *Trichosanthes kirilowii*

Shujiro Seo,*[a] Atsuko Uomori,[a] Yohko Yoshimura,[a] Ushio Sankawa,[b] Yutaka Ebizuka,[b] Haruo Seto,[c] and Ken'ichi Takeda[a]

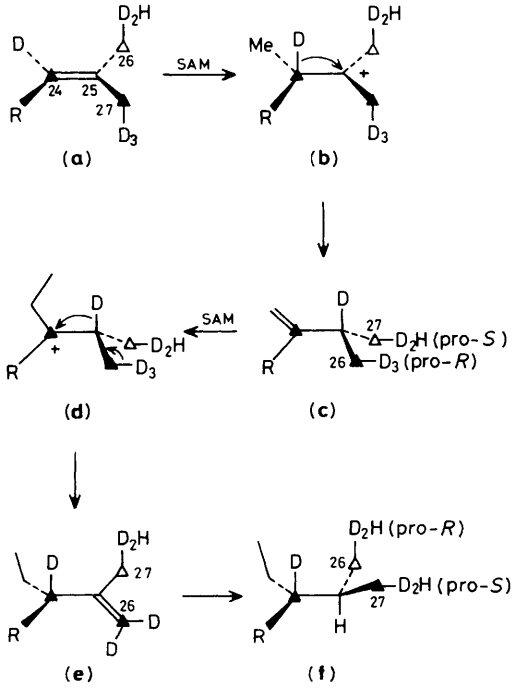
[a] *Shionogi Research Laboratories, Shionogi & Co. Ltd., Fukushima-ku, Osaka 553, Japan*
[b] *Faculty of Pharmaceutical Sciences, University of Tokyo, Hongo, Bunkyo-ku, Tokyo 113, Japan*
[c] *Institute of Applied Microbiology, University of Tokyo, Yayoi, Bunkyo-ku, Tokyo 113, Japan*

In the biosynthesis of 22-dihydrochondrillasterol (**4**) from [2-^{13}C,2-$^{2}H_3$]acetate and [1,2-$^{13}C_2$]acetate in cell cultures of *Trichosanthes kirilowii*, the hydrogen atom coming from C-4 of mevalonic acid was revealed by ^{13}C n.m.r. spectroscopy to be located at C-24, and that the protonation at C-25 of the 24β-ethyl-Δ^{25} precursor, to form the saturated side chain of (**4**), was most likely to occur from the *Re*-face.

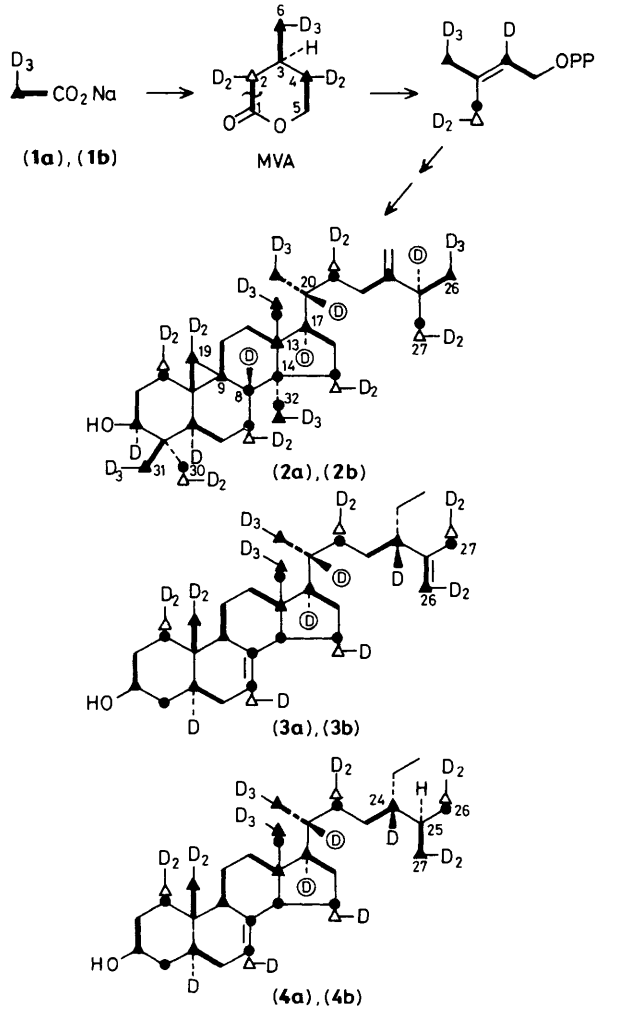
The biosynthesis of phytosterols from acetic acid and mevalonic acid (MVA) in photosynthetic organisms has been well documented[1] and the evolution of the alkylation mechanism to achieve different chiralities of the alkyl group at C-24 is becoming better understood.[2]

Recently, we confirmed by ^{13}C n.m.r. spectroscopy, in cell cultures of higher plants, the distribution of carbon and hydrogen atoms originating from acetic acid in the biosynthesis of a 24α-ethylsterol, sitosterol, and found that the 24-H of cycloartenol, originating from 4-H of MVA, was lost during the side chain formation.[3]

The seeds of some *Cucurbitaceae* plants, such as *C. pepo* and *Trichosanthes kirilowii*, are known to contain principally 24β-ethylsterols.[4] However, tissues from the mature plants mainly synthesize 24α-ethylsterols, the highly evolved forms.[5] This apparent evolutionary recapitulation during the development of plants prompted us to investigate the mechanism for alkylation at C-24. The callus, which we induced from the aerial parts of *T. kirilowii* Maxim. var. *japonica*, produced mainly the 24β-ethylsterols, 22-dihydrochondrillasterol (**4**) and 22-dihydro-25-dehydrochondrillasterol† (**3**). The mechanism of side-chain formation of the 24β-ethylsterol, poriferasterol, has been studied in *Ochromonas malhamensis*,



Scheme 1. Proposed mechanism for 24β-ethyl side-chain formation. ▲: Carbon derived from C-4 and C-6 of MVA, △: carbon derived from C-2 of MVA. SAM = S-adenosyl methionine.



Scheme 2. **a**: △, ▲ indicate the carbon from C-2 of [2-^{13}C, 2-D_3] acetate (**1a**) and, more specifically, △ also shows the carbon from C-2 of MVA. **b**: ●, ━ indicate singly and doubly labelled carbon from [1,2-$^{13}C_2$] acetate (**1b**), respectively.

† Nomenclature: 22-dihydrochondrillasterol (**4**) = (24*S*)-24-ethyl-5α-cholest-7-en-3β-ol; 22-dihydro-25-dehydrochondrillasterol (**3**) = (24*S*)-24-ethyl-5α-cholesta-7,25-dien-3β-ol; 24-methylenecycloartanol (**2**) = 9,19-cyclo-4,4,14,24-tetramethyl-5α,9β,14α-cholest-24(28)-en-3β-ol.

Table 1. ^{13}C N.m.r. data[a] for 24-methylenecycloartanol (**2**), 22-dihydro-25-dehydrochondrillasterol (**3**), and 22-dihydrochondrillasterol (**4**)[b] biosynthesized from [2-^{13}C,2-$^{2}H_3$]acetate (**1a**) and [1,2-$^{13}C_2$]acetate (**1b**) in tissue cultures of *Trichosanthes kirilowii* Maxim. var. *japonica*.

	(**2b**)		(**2a**)			(**3b**)		(**3a**)			(**4b**)		(**4a**)		
	δ_C	J_{CC}	$^1\Delta\delta_C(^2H)$			δ_C	J_{CC}	$^1\Delta\delta_C(^2H)$			δ_C	J_{CC}	$1\Delta\delta_C(^2H)$		
			d_1	d_2	d_3			d_1	d_2	d_3			d_1	d_2	d_3
C-1[c]	31.98	s	−0.33	−0.77		37.15	s	−0.42	−0.80		37.16	s	[e]	−0.80	
			−0.44												
C-2	30.41	37				31.47	36				31.47	36			
C-3[c]	78.85	37	−0.54			71.05	36				71.05	36			
C-4	40.50	36				37.98	s				37.97	s			
C-5[c]	47.13	35	−0.64			40.26	34	−0.54			40.27	34	−0.55		
C-6	21.13	35				29.66	34				29.67	34			
C-7[c]	26.02[f]	s	−0.41	[e]		117.43	s	−0.34			117.43	s	−0.35		
C-8	48.00	s				139.60	s				139.59	s			
C-9[c]	20.02	43	(−0.09)[d]			49.46	34				49.47	34			
C-10	26.10	12				34.21	37				34.21	36			
C-11	26.50[f]	43				21.56	34				21.57	34			
C-12	32.92[f]	35				39.58	36				39.58	36			
C-13[c]	45.32	35	(−0.09)[d]			43.39	36	(−0.09)[d]			43.38	36	(−0.08)[d]		
C-14	48.83	s				55.04	s				55.05	s			
C-15[c]	35.58[f]	s	−0.39	−0.77		22.96	s	−0.35			22.99	s	−0.35		
C-16	28.17[f]	33				27.89	33				27.97	33			
C-17[c]	52.29	33	(−0.11)[d]			56.09	33	(−0.11)[d]			56.08	33	(−0.11)[d]		
C-18[c]	18.04	s	−0.31	−0.60	−0.85	11.84	s	−0.28	−0.57	−0.83	11.85	s	−0.31	−0.57	−0.88
C-19[c]	29.90	12	−0.44	−0.86		13.03	37	−0.26	−0.57		13.04	36	−0.31	−0.60	
C-20	36.13	35				35.99	35				36.73	35			
C-21[c]	18.32	35	[e]	[e]	[e]	18.77	35	−0.31	−0.61	[e]	18.98	35	−0.32	−0.61	−0.92
C-22[c]	35.02	s	−0.42	−0.80		33.63	s	−0.41	−0.82		33.88	s	−0.44	−0.83	
C-23	31.33	41				29.50	34				26.52	35			
C-24[c]	156.92	41	(−0.03)[d]			49.53	34	−0.53			46.07	35	−0.61		
C-25	33.82	35				147.53	72				28.97	35			
C-26[c]	22.01	35	−0.31	−0.60	[e]	111.39	72	−0.29	−0.56		19.61	s	−0.31	[e]	
C-27[c]	21.88	s	−0.31	−0.61		17.78	s	−0.28	−0.54		18.98	35	−0.32	−0.61	
C-28	105.94					26.53					23.01				
C-29						12.07					12.33				
C-30[c]	25.45	s	−0.31	−0.61											
C-31[c]	14.01	36	−0.29	−0.57	−0.88										
C-32[c]	19.33	s	−0.29	−0.58	−0.93										

[a] ^{13}C-{^1H}{^2H} N.m.r. spectra were recorded on a JEOL GX-400 spectrometer in the ^1H and ^2H decoupling mode at 100 MHz in [^2H]chloroform (δ_C 77.000). J_{CC} values (in Hz) were obtained from ^{13}C-{^1H} n.m.r. spectra recorded on a Varian XL-200 n.m.r. spectrometer at 50.309 MHz in the ^1H decoupling mode in [^2H]chloroform using tetramethylsilane as an internal standard (δ_C 0). A figure in the J_{CC} columns indicates ^{13}C–^{13}C doubly labelled carbon and s indicates singly labelled carbon. Accuracies of δ_C and J_{CC} are ±0.03 p.p.m. and ±2 Hz, respectively. [b] This compound contained about 20% of 24α-epimer. [c] Carbons originating from C-2 of acetate. [d] These are $^2\Delta\delta_C(^2H)$ values. [e] These signals were not observed due to overlapping with other signals. [f] Reported assignments of these carbon signals on cycloartanol derivatives[9] are revised by 2D INADEQUATE ^{13}C n.m.r. method.

in which the hydride shift from C-24 to C-25 occurs on the *Si*-face of the Δ^{24} precursor double bond.[6]

Here we report on the stereospecific hydride shifts and protonation at C-25 throughout the 24β-ethyl side-chain formation of (**4**) in cell cultures of *T. kirilowii* fed with [2-^{13}C, 2-$^{2}H_3$]acetate and [1,2-$^{13}C_2$]acetate, based on the labelling pattern observed by ^{13}C n.m.r. spectroscopy.

The callus was induced from the aerial parts of *T. kirilowii* on a Linsmaier and Skoog medium fortified with 2,4-D‖ (10^{-6} M) and kinetin (0.02 p.p.m.), and subcultured every four weeks under the same conditions.

Sodium [2-^{13}C,2-$^{2}H_3$]acetate (**1a**)‡ and sodium [1,2-$^{13}C_2$]acetate (**1b**)§ were administered independently for 12 days to the suspension cultures of this callus. Compounds (**3**), (**4**), and 24-methylenecycloartanol† (**2**) were isolated from the cells and the labelling patterns were observed by 50 MHz ^{13}C-{^1H} and 100 MHz ^{13}C-{^1H}{^2H} n.m.r. spectroscopy. ^{13}C N.m.r. signal assignments are shown in Table 1. C-26 (the pro-*R* methyl group at C-25) and C-27 (the pro-*S* methyl group at C-25) of (**4**) were assigned by comparison with those of clionasterol in our previous report.[7]

As shown in Table 1, ^{13}C signals at C-24 in (**3a**) and (**4a**) (δ_C 49.52 and 46.07, respectively), accompanied the signals shifted by the α-deuterium effect [$^1\Delta\delta_C(^2H)$ −0.53 and −0.61]. These results indicate that the deuterium atom derived from C-4 of MVA is located at C-24. The plausible mechanism for 24β-ethyl side-chain formation deduced from our results is shown in Scheme 1. The first hydride (deuteride) shift (**a**) → (**b**) → (**c**) takes place on the *Re*-face of the original double bond and gives the side chain (**c**) in which the methyl group at C-25, originating from C-2 of MVA, becomes oriented to the pro-*S* position as reported by Arigoni for yeast.[8] The second methylation onto the $\Delta^{24(28)}$ double bond

‖ 2,4-D = 2,4-dichlorophenoxyacetic acid.

‡ 1014 mg of acetate in 7.8 l of medium.

§ A mixture of labelled acetate (714 mg) and non-labelled acetate (1428 mg) in 10.2 l of medium.

gives a cationic intermediate (**d**), and the hydrogen (deuterium) at C-25 migrates back to C-24. Then a hydrogen (deuterium) elimination from the methyl group at C-25 arising from C-6 of MVA gives the side-chain (**e**). The last reduction occurs at C-25 from the *Re*-face of the $\Delta^{25(26)}$ double bond and gives (**f**).

Evidence for this mechanism came from the following findings: (i) a β-deuterium shifted signal at C-24 [δ_C 156.92, $^2\Delta\delta_C(^2H)$ −0.03] of (**2a**); (ii) the pro-*S* (C-27) and pro-*R* (C-26) methyls at C-25 of (**2b**) appearing as a singlet and a doublet (*J* 35 Hz), respectively; (iii) (**3a**) having C-27 labelled by $^{13}CD_2H$ [δ_C 17.78, $^1\Delta\delta_C(^2H)$ −0.54]; (iv) a singlet C-27 and a doublet C-26 (*J* 72 Hz) in (**3b**); (v) (**4b**) having a singlet C-26 (δ_C 19.61) and a doublet C-27 (δ_c18.98, *J* 35 Hz), the latter being labelled by $^{13}CD_2H$ in (**4a**). The two deuterium atoms at C-22 of (**3a**) and (**4a**) clearly indicate that these sterols were not formed primarily through a $\Delta^{22(23)}$precursor.

The ^2H and ^{13}C distributions in the tetracyclic skeleton of (**3**) and (**4**) were similar to those of the 24α-ethylsterol, sitosterol.[3] 1,2-Methyl migration (C-18 from C-14) and 1,2-hydride shifts (20-H from C-17 and 17-H from C-13) during the cyclization from epoxysqualene were confirmed by the singlet signals at C-14 and C-18 in (**2b**), (**3b**) and (**4b**), and by the β-deuterium isotopically shifted signals at C-17 and C-13 in (**2a**), (**3a**), and (**4a**). 1,2-Methyl migration (C-32 from C-8) and 1,2-hydride shift (8-H from C-9) were also verified by the singlets, C-8 and C-32, in (**2b**), and the β-deuterium isotopically shifted signal at C-9 [δ_C 20.02, $^2\Delta\delta_C(^2H)$ −0.09] of (**2a**). Thus, the 8-^2H observed in (**2a**) is evidence for cycloartenol, not lanosterol, being the primary cyclization product from epoxysqualene. The two deuterium atoms at C-19 of (**3a**) and (**4a**) agree with the intermediacy of cycloartenol. The retention of 5-^2H in (**3a**) and (**4a**) demonstrate that Δ^7 was not formed *via* a $\Delta^{5,7}$ precursor.

We thank Dr. Y. Nanjo of Aburahi Laboratories, Shionogi & Co., Ltd., for supplying the *T. kirilowii* plants and Dr. H. Tada of the same company for encouragement.

Received, 13th August 1987; Com. 1194

References

1 See reviews such as T. W. Goodwin, 'New Comprehensive Biochemistry,' eds. A. Neuberger and L. L. M. Van Deener, Elsevier, Amsterdam, vol. 12, 1985, ch. 7; T. W. Goodwin, 'Biosynthesis of Isoprenoid Compounds,' eds. J. W. Porter and S. L. Spurgeon, Wiley, New York, vol. 1, 1981, p. 443; E. Caspi, *Tetrahedron*, 1986, **42**, 3.
2 D. M. Harrison, *Natural Product Reports*, 1985, **2**, 526; S. Seo, A. Uomori, Y. Yoshimura, and K. Takeda, *J. Chem. Soc., Chem. Commun.*, 1984, 1174; A. Uomori, S. Seo, Y. Yoshimura, and K. Takeda, *ibid.*, 1984, 1176; L. Cattel, G. Balliano, F. Viola, and O. Caputo, *Planta Medica*, 1980, **38**, 112.
3 S. Seo, U. Sankawa, H. Seto, A. Uomori, Y. Yoshimura, Y. Ebizuka, H. Noguchi, and K. Takeda, *J. Chem. Soc., Chem. Commun.*, 1986, 1139.
4 W. Sucrow, M. Slopianka, and H. W. Kircher, *Phytochemistry*, 1976, **15**, 1533; T. Akihisa, S. Thakur, F. U. Rosenstein, and T. Matsumoto, *Lipids*, 1986, **21**, 39.
5 W. R. Nes, K. Krevitz, J. Joseph, W. D. Nes, B. Harris, and G. F. Gibbons, *Lipids*, 1977, **12**, 511; T. Itoh, K. Yoshida, T. Tamura, and T. Matsumoto, *Phytochemistry*, 1982, **21**, 727.
6 F. Nicotra, F. Ronchetti, G. Russo, L. Toma, P. Gariboldi, B. M. Ranzi, *J. Chem. Soc., Chem. Commun.*, 1984, 383; F. Nicotra, F. Ronchetti, G. Russo, L. Toma, and B. M. Ranzi, *Mag. Reson. Chem.*, 1985, **23**, 134.
7 S. Seo, A. Uomori, Y. Yoshimura, and K. Takeda, *J. Am. Chem. Soc.*, 1983, **105**, 6343.
8 D. Arigoni, 'Molecular Interaction and Activity in Protein,' Chiba Found Symp., 1978, **60**, 243; D. Arigoni, personal communication.
9 F. Khuong-Huu, M. Sangare, V. M. Chari, A. Bekaert, M. Devys, M. Barbier, and G. Lukacs, *Tetrahedron Lett.*, 1975, 1787.