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CONVERSION OF 24-METHYLCHOLESTEROL TO 6-OXO-24-METHYLCHOLESTANOL, A PUTATIVE INTERMEDIATE OF THE BIOSYNTHESIS OF BRASSINOSTEROIDS, IN CULTURED CELLS OF *CATHARANTHUS ROSEUS*

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Key Word Index—*Catharanthus roseus*; brassinosteroids; biosynthesis; 6-oxo-24-methylcholestanol (3β-hydroxy-24-methyl-5α-cholestan-6-one); 6α-hydroxy-24-methylcholestanol (24-methyl-5α-cholestane-3β,6α-diol).

Abstract—Early steps of brassinosteroid biosynthesis were investigated using cultured cells of *Catharanthus roseus*. In the cells, 24-methylcholesterol was the major sterol component which accounted for 50% of total sterols and was assumed to be the possible biosynthetic precursor of brassinolide. [$^{13}C_5$] and/or [^{14}C]24-methylcholesterol were prepared by the cultured cells in the presence of compactin, a mevalonic acid biosynthetic inhibitor. The endogenous metabolites of the labelled 24-methylcholesterol, 24-methylcholestanol, 6α-hydroxy-24-methylcholestanol (24-methyl-5α-cholestane-3β,6α-diol), and 6-oxo-24-methylcholestanol (3β-hydroxy-24-methyl-5α-cholestan-6-one) were identified by full-scan GC–mass spectrometry. The biosynthetic sequence of 24-methylcholesterol → 24-methylcholestanol → 6α-hydroxy-24-methylcholestanol → 6-oxo-24-methylcholestanol was established in cultured cells of *C. roseus* by feeding experiments using labelled 24-methylcholestanol and 6α-hydroxy-24-methylcholestanol. This sequence probably constitutes an early part of the biosynthetic pathway of brassinolide, as introduction of vicinal hydroxyls in the side chain (C-22*R* and C-23*R*) of 6-oxo-24-methylcholestanol will yield teasterone. This is the first report of the natural occurrence of 6α-hydroxy-24-methylcholestanol.

INTRODUCTION

Brassinosteroids represent a new class of plant hormone with wide occurrence in the plant kingdom and unique biological activities on plant growth [2]. Although brassinosteroids elicit strong growth-promoting activity in many plant systems, their physiological functions in plants are not fully understood to date. Establishment of their biosynthetic pathway and subsequent studies on biosynthetic inhibitors and regulation of their biosynthesis will provide important clues to their physiological functions.

We have studied the biosynthesis of brassinosteroids, and have recently established that brassinolide (**6**), the most biologically active and widely occurring natural brassinosteroid, is biosynthesized from teasterone (**5**) via typhasterol and castasterone in cultured cells [3–5] and intact seedlings [6] of *Catharanthus roseus*. In the seedlings of *Nicotiana tabacum* and *Oryza sativa*, teasterone (**5**) was converted to castasterone via typhasterol [6]. In addition, possible involvement of 3-dehydroteasterone in the conversion of teasterone (**5**) to typhasterol was shown in several plant systems [1, 7, 8]. Therefore, the above pathway leading to brassinolide (**6**) appears to be ubiquitous in the plant kingdom. The biosynthetic pathway of brassinosteroids preceding teasterone (**5**), however, is still unknown. Among the common sterols, campesterol (24α-methylcholesterol of **1b**) or its analogues are assumed to be the biosynthetic precursors of brassinolide (**6**) based on the similarity of the side-chain structure.

In this study, we first investigated the principal sterols in cultured cells of *C. roseus* in order to discover possible steroidal precursors in brassinolide (**6**) biosynthesis. We then prepared labelled sterols by feeding labelled mevalonic acid (MVA) to the cultured cells of *C. roseus* in

Biosynthesis of Brassinosteroids in *Catharanthus roseus*, Part V. For Part IV, see ref. [1].

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Table 1. Identification of principal sterols in *Catharanthus roseus* cultured cells V208

Identified sterol	Relative R_t on GC*	Principal ions [*m/z* (rel. int.)]	Relative content (%)
Cholesterol (cholest-5-en-3β-ol, **1a**)	1.000	458 (M^+, 12), 443 (7), 368 (32), 353 (32), 329 (100), 255 (40), 213 (34)	2
24-Methylcholesterol (24-methylcholest-5-en-3β-ol, **1b**)	1.032	472 (M^+, 13), 457 (7), 382 (57), 367 (32), 343 (100), 255 (42), 213 (38)	49
24-Ethyl-22*E*-dehydrocholesterol (24-ethylcholesta-5,22-dien-3β-ol, **1c**)	1.041	484 (M^+, 10), 469 (3), 394 (29), 379 (36), 355 (30), 312 (21), 255 (100), 213 (47)	11
24-Ethylcholesterol (24-ethylcholest-5-en-3β-ol, **1d**)	1.059	486 (M^+, 14), 471 (5), 396 (72), 381 (39), 357 (100), 255 (58), 213 (43)	26
24-Methylcholestanol (24-methyl-5α-cholestan-3β-ol, **2b**)	1.034	474 (M^+, 7), 459 (11), 417 (4), 384 (8), 369 (16), 305 (18), 257 (9)	8
24-Ethyl-22*E*-dehydrocholestanol (24-ethyl-5α-cholest-22-en-3β-ol, **2c**)	1.044	486 (M^+, 14), 471 (13), 429 (2), 396 (4), 381 (26), 374 (13), 353 (32), 345 (25), 257 (100)	1
2c acetate	1.044†	456 (M^+, 31), 353 (34), 344 (34), 315 (46), 257 (100), 255 (47), 215 (38)	
24-Ethylcholestanol (24-ethyl-5α-cholestan-3β-ol, **2d**)	1.066	488 (M^+, 8), 473 (11), 431 (5), 398 (9), 383 (16), 305 (21), 257 (9)	3

Sterols were identified by GC–mass spectrometry as TMSi derivatives under the conditions described in Experimental.
*R_t relative to cholesterol-TMSi.
†R_t relative to cholesterol acetate.

the presence of a MVA biosynthetic inhibitor (compactin). These experiments yielded metabolites that were assumed to be intermediates of brassinolide (**6**) biosynthesis. Identification of these metabolites and studies of their metabolic sequence using chemically synthesized labelled compounds are also described.

RESULTS

Identification of endogenous sterols

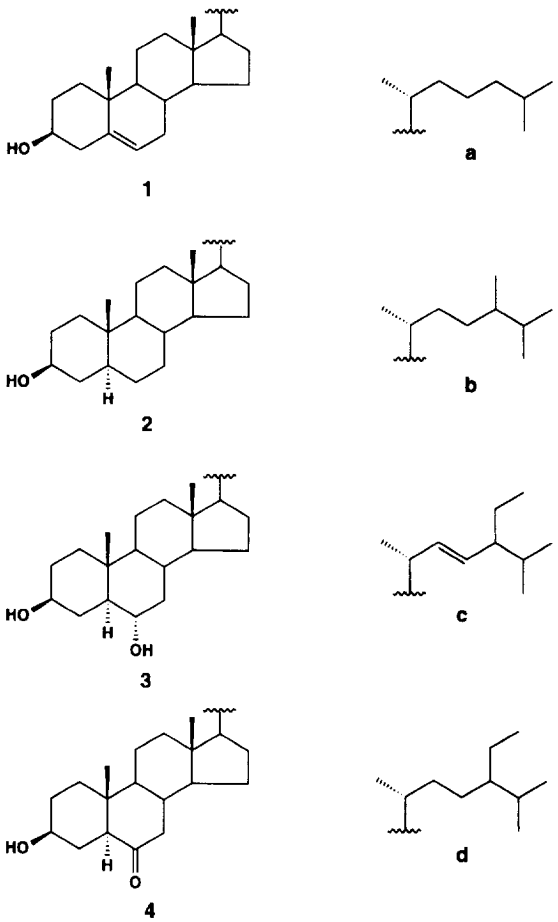
The sterol fraction obtained from the cultured cells of *C. roseus* (V208) as described in the Experimental section was derivatized and analysed by GC–mass spectrometry. As shown in Table 1, four common sterols, namely cholesterol (**1a**), 24-methylcholesterol (**1b**), 24-ethyl-22*E*-dehydrocholesterol (**1c**) and 24-ethylcholesterol (**1d**), and two B-ring saturated sterols of 24-methylcholestanol (**2b**) and 24-ethylcholestanol (**2d**) were identified by GC–mass spectrometry by direct comparison with the authentic samples. Identification of 24-ethyl-22*E*-dehydrocholestanol (**2c**) was based on good agreement of the mass spectrum of its acetate with reported data [9]. Among the C_{28} sterols which are considered to be possible biosynthetic precursors of brassinolide (**6**), 24-methylcholesterol (**1b**) was found to constitute the major component, accounting for *c.* 50% of the total sterol content. The NMR studies showed that the endogenous sterol was an epimeric mixture at C-24 (24α:24β = 4:1), and therefore it was called 24-methylcholesterol instead of campesterol.

Preparation of labelled sterols

Based on the above results, ^{14}C-labelled and ^{13}C-labelled 24-methylcholesterol were prepared by feeding ^{14}C-labelled and ^{13}C-labelled MVA to cultured cells of *C. roseus*. A biosynthetic inhibitor of MVA was used in order to avoid the dilution by endogenous sterols.

Compactin has been developed as an inhibitor of 3-hydroxy-3-methylglutaryl CoA reductase, which catalyses the formation of MVA [10]. The growth inhibition of intact plants and cultured cells caused by the inhibitors of MVA biosynthesis has been found to be regained by the simultaneous application of MVA [11–17]. The growth of *C. roseus* cells which was inhibited by 40 μM of compactin completely recovered after simultaneous application of a 3 mM or greater concentration of MVA. Quantification of sterols by GC–mass spectrometry revealed that the content of endogenous sterols was also decreased by the application of compactin in a concentration-dependent manner and that this decrease recovered after simultaneous application of MVA. Thus, compactin inhibited the biosynthesis of MVA resulting in the inhibition of both cell growth and sterol biosynthesis, and simultaneous application of MVA reversed both of these inhibitions.

From these results, application of labelled MVA with compactin to the cultured cells would give labelled sterols with reduced dilution by endogenous sterols. Therefore, labelled sterols used for feeding experiments were prepared by incubating V208 cells with 40 μM of compactin and 5 mM of [^{13}C]MVA. After culture, sterols were extracted and purified by silica gel HPLC and then separated into each sterol fraction by ODS-HPLC. On GC–mass spectrometry analysis, the sample from 24-methylcholesterol fraction showed a single peak at the same retention time as authentic 24-methylcholesterol (**1b**) and its mass spectrum indicated that the 24-methylcholesterol obtained from the cells was [$^{13}C_5$]24-methylcholesterol, with little dilution by endogenous



24-methylcholesterol (**1b**), and was labelled at C-1, C-7, C-15, C-22, and C-26 as expected from sterol biosynthetic pathway. [$^{13}C_5$]24-Methylcholestanol was also obtained from the sterol fraction of the above experiment. A mixture of [^{13}C]- and [^{14}C]24-methylcholesterol (specific activity, 208.0 MBq mmol^{-1}) was prepared in similar manner using compactin and a mixture of [^{13}C]- and [^{14}C]MVA.

Metabolism of [^{14}C]*24-methylcholesterol*

In order to find intermediates in brassinolide (**6**) biosynthesis derived from 24-methylcholesterol (**1b**), feeding experiments of labelled 24-methylcholesterol were carried out using V208 cell line.

Preliminarily, metabolism of [^{14}C]24-methylcholesterol in V208 cells was investigated. [^{14}C]24-Methylcholesterol (3.3 kBq) prepared without compactin was fed to 9-day-old V208 cells. After 3 days of culture, metabolites were purified by solvent fractionation, treatment with an ODS cartridge column, and ODS-HPLC. Radioactivity in the metabolites was detected in two fractions, which were analysed by GC–mass spectrometry after derivatization. From the fraction with R_t 20–21 min, 24-methylcholestanol (**2b**) was identified by direct comparison of the mass spectrum with that of an authentic sample. From the R_t 7–11 min fraction, two steroidal compounds were detected by GC–mass spectrometry as possible intermediates of brassinolide (**6**) biosynthesis. One was assumed to be an oxo-derivative of 24-methylcholestano (**2b**) from its mass spectrum, and identified as 6-oxo-24-methylcholestanol (**4b**) by direct comparison of the mass spectrum with that of authentic sample (Table 2) prepared chemically from 24-methylcholesterol (**1b**), as described in the Experimental. The other metabolite was determined from its mass spectrum to be a hydroxylated derivative of 24-methylcholestanol (**2b**) at C-6. 6α-(**3b**) and 6β-hydroxy-24-methylcholestanols were prepared from 6-oxo-24-methylcholestanol (**4b**) by Birch reduction and by reduction with $NaBH_4$, respectively. These two hydroxy-24-methylcholestanols afforded different retention times on GC and different mass spectra. The metabolite of 24-methylcholesterol (**1b**) was found to be identical with 6α-hydroxy-24-methylcholestanol (**3b**) by comparison of its GC–mass spectrometry data (Table 2). The endogenous contents of **2b**, **3b** and **4b** in the cells were roughly estimated from GC–mass spectrometry data to be approximately 40 μg g fr. wt^{-1}, 2 μg g fr. wt^{-1}, < 1 μg g fr. wt^{-1}, respectively.

Metabolism of [$^{13}C_5$]*24-methylcholesterol*

V208 cells (9-day-old) were fed with a mixture of [$^{13}C_5$]24-methylcholesterol (50 μg) and [^{14}C]24-methyl-

Table 2. Identification of possible intermediates of brassinolide biosynthesis in *Catharanthus roseus* cultured cells V208

Identified sterol	Relative R_t on GC*	Principal ions [*m/z* (rel. int.)]
24-Methylcholestanol (24-methyl-5α-cholestan-3β-ol, **2b**)	1.034	474 (M$^+$, 7), 459 (11), 417 (4), 384 (8), 369 (16), 305 (18), 257 (9)
6α-Hydroxy-24-methylcholestanol (24-methyl-5α-cholestane-3β,6α-diol, **3b**)	1.078	562 (M$^+$, 34), 547 (32), 472 (68), 457 (31), 417 (28), 383 (72), 382 (68)
6-Oxo-24-methylcholestanol (3β-hydroxy-24-methyl-5α-cholestan-6-one, **4b**)	1.121	488 (M$^+$, 20), 473 (57), 459 (100)

Sterols were identified by GC mass spectometry as TMSi derivatives under the conditions described in Experimental.
*R_t relative to cholesterol-TMSi.

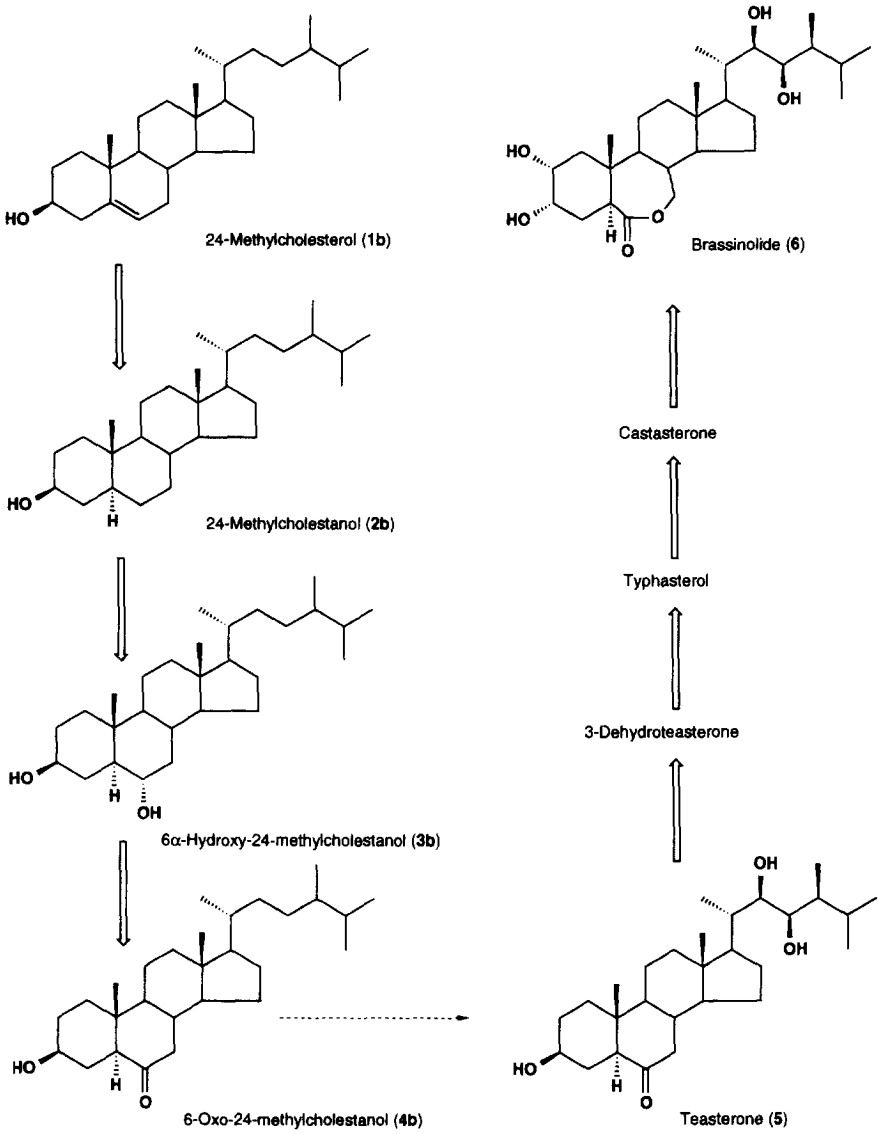


Fig. 1. Proposed biosynthetic pathway of brassinolide from 24-methylcholesterol in cultured cells of *Catharanthus roseus*. ⇒: established by feeding experiments (refs 1, 3–5). ---> : not established in this study.

cholesterol (3.3 kBq), and incubated for 3 days. The partially purified extract of the cells was analysed by ODS-HPLC, and the radioactive fractions were analysed by GC–mass spectrometry after derivatization. Endogenous 24-methylcholestanol (**2b**) was identified from the R_t 20–21 min, fraction. The isotopic ions in the mass spectrum indicated the presence of [$^{13}C_5$]24-methylcholestanol and, therefore, 24-methylcholestanol (**2b**) was converted from 24-methylcholesterol (**1b**). From the R_t 7–11 min fraction, 6α-hydroxy-24-methylcholestanol (**3b**) and 6-oxo-24-methylcholestanol (**4b**) were identified as endogenous compounds. Isotopic ions resulting to $^{13}C_5$-labelled compounds were clearly observed in these spectra. Thus, these compounds were confirmed to be derived from 24-methylcholesterol (**1b**).

Metabolism of [$^{13}C_5$]*24-methylcholestanol and* [$^2H_6$]*6α-hydroxy-24-methylcholestanol*

24-Methylcholestanol (**2b**), 6α-hydroxy-24-methylcholestanol (**3b**), and 6-oxo-24-methylcholestanol (**4b**) were found to be metabolites of 24-methylcholesterol (**1b**) and could be intermediates leading to teasterone (**5**). In order to confirm the biosynthetic sequence of these compounds, feeding experiments were conducted with V208 cells using [$^{13}C_5$]24-methylcholestanol obtained biosynthetically as described above and [$^2H_6$]6α-hydroxy-24-methylcholestanol prepared chemically from [$^2H_6$]6-oxo-24-methylcholestanol. On GC–SIM analysis of the 6α-hydroxy-24-methylcholestanol (**3b**) fraction of the V208 cells fed with [$^{13}C_5$]24-methylcholestanol, isotopic

ions arising from [$^{13}C_5$]6α-hydroxy-24-methylcholestanol (*m/z* 567 $[M]^+$ and 477 $[M - 90]^+$) were clearly detected along with ions of endogenous 6α-hydroxy-24-methylcholestanol (*m/z* 562 $[M]^+$ and 472 $[M - 90]^+$). Similarly, on GC–SIM analysis of the 6-oxo-24-methylcholestanol (**4b**) fraction of the cells fed with [$^2H_6$]6α-hydroxy-24-methylcholestanol, isotopic ions arising from [$^2H_6$]6-oxo-24-methylcholestanol (*m/z* 494 $[M]^+$ and 465 $[M - 29]^+$) were detected with ions due to endogenous 6-oxo-24-methylcholestanol (*m/z* 488 $[M]^+$ and 459 $[M - 29]^+$).

Thus, the biosynthetic sequence of 24-methylcholesterol (**1b**) → 24-methylcholestanol (**2b**) → 6α-hydroxy-24-methylcholestanol (**3b**) → 6-oxo-24-methylcholestanol (**4b**) was established in the cultured cells of *C. roseus* (Fig. 1).

DISCUSSION

Sterol analysis of *C. roseus* (V208) cells revealed that 24-methylcholesterol (**1b**) was the major (50%) component of the sterol fraction (Table 1). In higher plants, 24-ethylcholesterol (**2d**) is known to be the major component of the sterol fraction [18]. The seed oil of *C. roseus* was reported to contain 24-ethylcholesterol (**2d**; 38.5%) as the major sterol, while 24-methylcholesterol (**1b**) accounted for 24.4% of the total sterol content [19]. The B-ring saturated sterols found in the cultured cells of *C. roseus* have not been identified in the seed oils. Conversely, isofucosterol and 24-methylenecholesterol found in the seeds were not identified in the cultured cells used in this study. Thus, the cultured cells of *C. roseus* were found to be different from the mother plant in sterol composition. There is no evidence whether this characteristic sterol composition is related to the production of brassinosteroids by the cells. However, the cultured cells of *C. roseus* were useful both for studying the biosynthesis of brassinolide (**6**), and also for the preparation of labelled 24-methylcholesterol, which was assumed to be a primary precursor of brassinolide (**6**).

In the biosynthetic preparation of labelled sterols from labelled MVA by the cultured cells, the use of compactin was very effective for avoiding the dilution of labelled compounds with endogenous ones: there was practically no dilution in the case of $^{13}C_5$-labelled sterols. Effective incorporation of labelled MVA to sterols has been reported in the cultured cells of sunflower [20]. However, dilution by endogenous sterols in our studies on the cultured cells of *C. roseus* was inevitable. The preparation of highly concentrated labelled sterols by this procedure was in pursuit of the minor metabolites in the biosynthetic pathway of brassinolide (**6**).

From the cultured cells of *C. roseus* (V208), 24-methylcholestanol (**2b**), 6α-hydroxy-24-methylcholestanol (**3b**), and 6-oxo-24-methylcholestanol (**4b**) were identified by GC–mass spectrometry as endogenous compounds and also as metabolites of 24-methylcholesterol (**1b**). 24-Methylcholestanol (**2b**), along with 24-ethylcholestanol (**2d**) are known to occur as minor components in the shoots of *Zea mays* [21], the tubers of *Dioscorea batatas* [22], and the shoots of *Arabidopsis thaliana* [23]. 6-Oxo-24-methylcholestanol (**4b**) and 6-oxo-24-ethylcholestanol (**4d**) were recently identified as minor sterols from *Mandevilla pentlandiana* [24]. However, this is the first report of the natural occurrence of 6α-hydroxy-24-methylcholestanol (**3b**).

6-Oxo-24-methylcholestanol (**4b**) was confirmed to be biosynthesized from 24-methylcholesterol (**1b**) via 24-methylcholestanol (**2b**) and 6α-hydroxy-24-methylcholestanol (**3b**) and is a promising candidate as a precursor in brassinolide (**6**) biosynthesis (Fig. 1). The conversion of 6-oxo-24-methylcholestanol (**4b**) to teasterone (**5**) involves the formation of vicinal diol in the side chain. The stereospecific introduction of diol functionality may take place by stepwise hydroxylation at C-22 and C-23, or through 22,23-epoxidation and subsequent hydration of the epoxide ring.

EXPERIMENTAL

Cell line and culture condition. Crown gall cells (V208) and nontransformed cells (Vn) of *C. roseus* were cultured as previously described [4].

Authentic sterols. 24-Methylcholesterol (**1b**) and 24-ethylcholesterol (**1d**) were purchased from Tama Biochemical Co., Ltd. 24-Ethyl-22*E*-dehydrocholesterol (**1c**) and cholesterol (**1a**) were obtained from Sigma. 24-Methylcholestanol (**2b**) and 24-ethylcholestanol (**4d**) were obtained by catalytic hydrogenation (Pd-C) of 24-methylcholesterol (**1b**) and 24-ethyl-22*E*-dehydrocholesterol (**1c**), respectively.

GC–MS and NMR analyses. GC–MS analysis was carried out on a Finnigan MAT INCOS 50 mass spectrometer connected to a Hewlett–Packard 5890A gas chromatograph with a capillary column DB-1 (0.258 mm × 15 m, 0.25 μm film thickness) or on a JEOL Automass JMS-AM150 mass spectrometer connected to a Hewlett–Packard 5890A-II gas chromatograph with a capillary column TC-1 (0.25 mm × 15 m, 0.25 μm film thickness). The temperature conditions of the GC was as previously reported [5]. Samples were treated with *N,O*-bis(trimethylsilyl)acetamide for 30 min at 70° prior to injection. Mass spectra of the synthetic compounds were measured with a JEOL Automass JMS-AM150 mass spectrometer by direct inlet EI mode. High-resolution mass spectra were measured with a JEOL AX-505 mass spectrometer. ^1H NMR spectra were recorded with a Bruker AC-P300 spectrometer (300 MHz) using $CDCl_3$ as a solvent and TMS as an internal standard.

*Preparation of 6-oxo-24-methylcholestanol (**4b**), and 6α-(**3b**) and 6β-hydroxy-24-methylcholestanols.* These compounds were prepared from 3β-acetoxy-24-methyl-5α-cholestan-6-one which was synthesized according to the method of Takatsuto and Ikekawa [25] as follows.

24-Methylcholesteryl tosylate (349 mg), prepared from 24-methylcholesterol (**1b**) and *p*-toluenesulfonyl chloride was treated with $KHCO_3$ in aq. Me_2CO to yield iso-24-methylcholesterol (219 mg). This was oxidized with Jones

reagent in acetone to give iso-24-methylcholesterone (137 mg), which was refluxed with HOAc and 5 M H_2SO_4 to give 3β-acetoxy-24-methyl-5α-cholestan-6-one (82 mg, 28%). NMR δH: 0.66 (3H, *s*, H-18), 0.77 (3H, *s*, H-19), 0.78 (3H, *d*, $J = 5.9$ Hz), 0.80 (3H, *d*, $J = 6.9$ Hz), 0.85 (3H, *d*, $J = 6.8$ Hz), 0.91 (3H, *d*, $J = 6.5$ Hz, H-21), 2.02 (3H, *s*, H-3β-acetoxy), 2.26 (1H, *dd*, $J = 12.8$ and 2.8 Hz), 2.32 (1H, *dd*, $J = 13.1$ and 4.5 Hz, H-7), 4.67 (1H, *tt*, $J = 11.6$ and 1.8 Hz, H-3α). MS *m/z* (rel. int.): 458 (M^+, 13), 398 (100), 383 (33), 380 (9), 286 (6), 271 (10), 244 (9), 229 (20), 211 (14), 185 (12), 181 (11), 161 (13), 159 (11).

A mixture of 3β-acetoxy-24-methyl-5α-cholestan-6-one (25 mg) in MeOH (4 ml) and 10 M aq. NaOH (1 ml) was refluxed overnight. The reaction mixture was diluted with H_2O and neutralized with 2 M aq. HCl. After removal of MeOH by evaporation, the residue was extracted with EtOAc. The extract was dried over dry Na_2SO_4 and concentrated to give 6-oxo-24-methylcholestanol [3β-hydroxy-24-methyl-5α-cholestan-6-one, **4b**] (12 mg, 53%). NMR δH: 0.66 (3H, *s*, H-18), 0.76 (3H, *s*, H-19), 0.77 (3H, *d*, $J = 6.5$ Hz), 0.80 (3H, *d*, $J = 7.0$ Hz), 0.85 (3H, *d*, $J = 6.8$ Hz), 0.91 (3H, *d*, $J = 6.4$ Hz, H-21), 2.20 (1H, *dd*, $J = 12.4$ and 2.5 Hz), 2.32 (1H, *dd*, $J = 13.1$ and 4.5 Hz, H-7), 3.57 (1H, *tt*, $J = 11.2$ and 4.8 Hz, H-3α). MS *m/z* (rel. int.): 416 (M^+, 46), 401 (5), 398 (8), 383 (9), 289 (12), 247 (16), 229 (8), 213 (6), 211 (6), 187 (7), 185 (11), 177 (11), 175 (12), 173 (9), 163 (14), 161 (20), 159 (15), 55 (100).

6β-Hydroxy-24-methylcholestanol. To a solution of 3β-acetoxy-24-methyl-5α-cholestan-6-one (17 mg) in THF (2 ml) was added $NaBH_4$ (150 mg), and the mixture was stirred for 3 hr at room temp. The reaction mixture was poured into 2 M aq. HCl and extracted with EtOAc. The extract was dried over dry Na_2SO_4 and concentrated to give 6β-hydroxy-24-methylcholestanol [24-methyl-5α-cholestane-3β-,6β-diol] (13 mg, 85%). NMR δH: 0.69 (3H, *s*, H-18), 0.77 (3H, *d*, $J = 6.5$ Hz), 0.80 (3H, *d*, $J = 7.0$ Hz), 0.85 (3H, *d*, $J = 6.8$ Hz), 0.90 (3H, *d*, $J = 6.4$ Hz, H-21), 1.03 (3H, *s*, H-19), 3.65 (1H, *tt*, $J = 10.9$ and 4.9 Hz, H-3α), 3.80 (1H, *br d*, $J = 2.3$ Hz, H-6α). MS *m/z* (rel. int.): 418 (M^+, 6), 400 (82), 385 (7), 382 (8), 255 (6), 246 (10),232 (27), 228 (10), 213 (25), 199 (9), 187 (10), 175 (10), 173 (13), 166 (11), 161 (20), 159 (19), 55 (100). HRMS: Found: 418.3834, $C_{28}H_{50}O_2$ requires: M^+, 418.3811.

6α-Hydroxy-24-methylcholestanol (**3b**). A solution of 3β-acetoxy-24-methyl-5α-cholestan-6-one (30 mg) in Et_2O (5 ml) was added to a solution of lithium (30 mg) in liquid ammonia (5 ml) cooled at −78° with dry ice–Me_2CO [26]. After the mixture was stirred for 30 min, solid NH_4Cl was added and the ammonia allowed to evaporate at room temp. The residue was dissolved in 1N aq. HCl and extracted with Et_2O. The extract was dried over dry Na_2SO_4 and concentrated to give 6α-hydroxy-24-methylcholestanol [24-methyl-5α-cholestane-3β,6α-diol, **3b**] (16 mg, 59%). NMR δH: 0.65 (3H, *s*, H-18), 0.77 (3H, *d*, $J = 6.5$ Hz), 0.80 (3H, *d*, $J = 7.1$ Hz), 0.82 (3H, *s*, H-19), 0.85 (3H, *d*, $J = 6.8$ Hz), 0.90 (3H, *d*, $J = 6.4$ Hz, H-21), 3.42 (1H, *td*, $J = 10.6$ and 3.4 Hz, H-6β), 3.58 (1H, *tt*, $J = 11.1$ and 4.9 Hz, H-3α). MS *m/z* (rel. int.): 418 (M^+, 22), 400 (34), 385 (12), 382 (6), 273 (6), 249 (10), 231 (38), 213 (24), 199 (9), 175 (10), 173 (10), 163 (15), 161 (21), 159 (17), 55 (100). HRMS: Found: 418.3790, $C_{28}H_{50}O_2$ requires: M^+, 418.3811.

Preparation of [26,28-$^2H_6$]*6-oxo-24-methylcholestanol and* [26,28-$^2H_6$]*6α-hydroxy-24-methylcholestanol.* [$^2H_6$]6-Oxo-24-methylcholestanol was obtained from [26,28-$^2H_6$] (22*E*,24*S*)-3β-acetoxy-24-methyl-5α-cholest-22-en-6-one [27] by saponification followed by catalytic hydrogenation (Pd-C). [$^2H_6$]6α-Hydroxy-24-methylcholestanol was prepared from [$^2H_6$]6-oxo-24-methylcholestanol by the same procedure as for 6α-hydroxy-24-methylcholestanol (**3b**). Structures of these labelled compounds were confirmed by comparison of the mass spectra with those of 6-oxo-24-methylcholestanol (**4b**) and 6α-hydroxy-24-methylcholestanol (**3b**).

Purification of endogenous sterols. V208 cells at late log-phase (8-day-old) were extracted with a mixture of $CHCl_3$ and MeOH (1:4). The extract was partitioned between $CHCl_3$ and H_2O, and the $CHCl_3$-soluble fraction was saponified with 1N KOH/MeOH. The resulting unsaponifiable fraction was extracted with $CHCl_3$ and charged onto a cartridge of silica gel (Sep-Pak Silica). The fraction eluted with $CHCl_3$ was collected and subjected to GC–MS analysis after derivatization.

Effect of compactin and MVA on the growth of cultured cells of C. roseus. Compactin (ML-236B) was converted to its sodium salt according to the reported method [28]. A solution of compactin was added aseptically through a sterile membrane to a 50-ml flask containing V208 cells grown for 2 days (0.2 g) in 15 ml of Murashige–Skoog (MS) medium. For the inhibition-recovery studies, (*R,S*)-MVA was dissolved in EtOH, added to a sterile flask, and the EtOH evaporated *in vacuo*. The medium was autoclaved, and compactin and V208 cells were added to the flask. The cells were grown for 13 days under the conditions described previously [4]. After culture, the cells were separated from the medium by filtration, weighed, and then extracted for sterol quantification.

Preparation of ^{14}C *and* ^{13}C*-labelled sterols.* As a preliminary experiment, [^{14}C]24-methylcholesterol was prepared by feeding [(*R*)-2-^{14}C]MVA (specific activity 2.1 GBq mmol^{-1}) to Vn cells (8-day-old) without using compactin. After 3 days of culture, the cells were extracted with a mixture of $CHCl_3$ and MeOH and the extract was partitioned between $CHCl_3$ and H_2O. $CHCl_3$-soluble fraction was purified with a cartridge of silica gel (Sep Pak Silica), which was eluted with $CHCl_3$. The eluate was subjected to HPLC on a silica gel column as follows: column, Senshu Pak Silica (150 × 4.6 mm); solvent, 1% iso-PrOH in *n*-hexane; flow rate, 1 ml min^{-1}; detection, UV 205 nm. The fraction corresponding to the retention time of authentic sterols (R_t 10–12 min) was collected. This fraction was dissolved in MeOH and passed through a cartridge of ODS (Sep Pak C_{18}), which was eluted with MeOH. The eluate was subjected to HPLC on an ODS column as follows: column, Senshu Pak ODS (150 × 4.6 mm); solvent, MeOH; flow rate, 0.8 ml min^{-1}; detection, UV 205 nm. The fractions corresponding to authentic 24-methylcholesterol (R_t

17.5–19.5 min) were collected and further purified by ODS-HPLC under the same conditions to give [^{14}C]24-methylcholesterol (specific activity, 50 MBq mmol^{-1}).

[$^{13}C_5$]24-Methylcholesterol and [$^{13}C_5$]24-methylcholestanol were prepared as follows. V208 cells (0.1 g) were cultured in a 50-ml flask containing 15 ml of MS medium, 40 μM compactin and 5 mM of [(*R,S*)-2-^{13}C]MVA and grown for 9 days. Propagated cells were transferred to four 50-ml flasks containing the same medium as above and cultured for 12 days to yield 25.5 g of cells. The cells were extracted with a mixture of $CHCl_3$ and MeOH, and the extract was purified in the same manner as the preliminary experiments through HPLC on a silica gel column and HPLC on an ODS column. The fractions corresponding to24-methylcholesterol (**1b**) and 24-methylcholestanol (**2b**) were collected. Rechromatography of each fraction under the same conditions gave 840 μg of [$^{13}C_5$]24-methylcholesterol and 120 μg of [$^{13}C_5$]24-methylcholestanol.

A mixture of [^{13}C] and [^{14}C]24-methylcholesterol was also prepared by incubating V208 cells in the flask containing MS medium (15 ml), compactin (40 μM) and a mixture of (*R*)-[2-^{14}C]MVA (0.88 μM; specific activity, 2.1 GBq mmol^{-1}) and (*R,S*)-[2-^{13}C]MVA (72.67 μM). Sterols were extracted and purified as described above to yield 42.4 kBq of ^{13}C,^{14}C-labelled 24-methylcholesterol (specific activity, 208.0 MBq mmol^{-1}), which were used for the confirmation of the metabolites by GC–MS.

Metabolism of labelled sterols. An acetone solution of labelled sterol was added to a 50-ml flask containing V208 cells grown for 9–10 days in 15 ml of MS medium and incubated for 3 days. The cells were extracted with a mixture of $CHCl_3$ and MeOH, and the extract was partitioned between $CHCl_3$ and H_2O. $CHCl_3$-soluble fraction was dissolved in MeOH and passed through a cartridge of ODS and then subjected to ODS-HPLC analysis. ODS-HPLC analysis was carried out under the same conditions described above and fractions were collected every 1 min. When ^{14}C-labelled substrates were used, radioactivity of each fraction was measured by a liquid scintillation counter (Packard 2000CA TRI-CARB). Each fraction corresponding to the metabolite was subjected to GC–MS analysis after derivatization.

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