BIOSYNTHESIS OF ANTITUMOR ANTIBIOTIC, CYTOGENIN

HIROYUKI KUMAGAI, MASAHIDE AMEMIYA, HIROSHI NAGANAWA†,
TSUTOMU SAWA†, MASAAKI ISHIZUKA* and TOMIO TAKEUCHI†

Institute for Chemotherapy, M.C.R.F.,
18-24 Motono, Miyamoto, Numazu-shi, Shizuoka 410-03, Japan
†Institute of Microbial Chemistry, M.C.R.F.,
3-14-23 Kamiosaki, Shinagawa-ku, Tokyo 141, Japan

(Received for publication September 20, 1993)

The biosynthesis of antitumor antibiotic cytogenin, 3-hydroxymethyl-6-methoxy-8-hydroxy-isocoumarin, was studied by feeding ^{14}C- or ^{13}C-labeled compounds to culture of the producing organism, *Streptoverticillium eurocidicum* MI43-37F11. ^{14}C-Acetate and ^{14}C-methionine were efficiently incorporated into cytogenin as precursors. ^{13}C NMR studies proved that the carbon skeleton of cytogenin is derived from pentaketide intermediate due to head-to-tail condensation of five acetate units and methyl group of 6-OCH_3 is derived from methionine. It was suggested that 3-hydroxymethyl and/or 6-methoxy group of cytogenin were metabolized by hydroxylation and/or methylation from three intermediates.

Cytogenin, isolated from cultured broth of *Streptoverticillium eurocidicum* MI43-37F11, showed potent antitumor activity against Ehrlich carcinoma by its immunomodulatory activity[1]. Radioisotope-labeled cytogenin with high specific activity was indispensable to study absorption, distribution, excretion, metabolism and bioavailability in preclinical studies. By feeding ^{14}C-acetate to the culture of the cytogenin-producing organism, however, we could not obtain radioactive cytogenin with high specific activity. To produce it efficiently, the biosynthesis of cytogenin was studied using various ^{14}C- or ^{13}C-labeled compounds as precursors. In this paper, we report the biosynthesis of cytogenin and metabolism of cytogenin intermediates by NMR spectroscopic analysis and HPLC techniques.

Results and Discussion

Firstly, a medium composition (described in Experimental section) was investigated to achieve a high incorporation ratio of radiolabeled precursors and the strain which produced high quantity of cytogenin was selected among single spores obtained from *S. eurocidicum* MI43-37F11.

As shown in Fig. 1-A, compounds **II** and **III** were observed as major and minor compounds at 20th hour cultivation. On the other hand, cytogenin (**I**) could not be observed. At 30th hour cultivation, another compound **IV** was observed (Fig. 1-B). Besides cytogenin and its intermediates, an unknown peak at 5.3 minutes of retention time was observed. Since UV spectrum of the peak was remarkably different from that of isocoumarins, the substance observed at 5.3 minutes of retention time was decided to be not a cytogenin related compound. Fig. 2 shows the time course of cytogenin production as determined by HPLC in the cultured broth. Cytogenin was gradually increased in the cultured broth at the expense of **II**, **III** and **IV**. In the time course experiment, other cytogenin related compounds were not found. Therefore, the structures of **II**, **III** and **IV** were determined. The ultraviolet spectra of **II**, **III** and **IV** were almost identical to that of cytogenin which gave absorption maxima at 244 and 330 nm, respectively, in

Fig. 1. HPLC analysis of cytogenin (**I**) and its related compounds (**II**, **III** and **IV**) in cultured broth.

A; cultured for 20 hours, B; cultured for 30 hours, C; cultured for 45 hours.

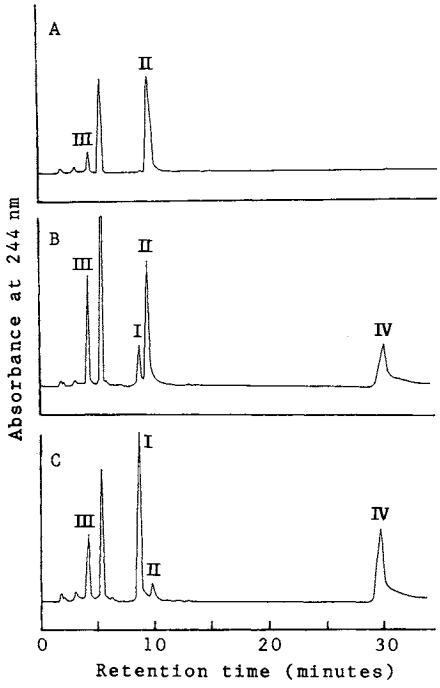
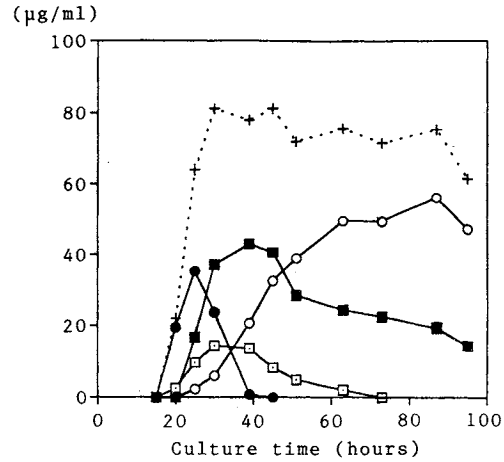


Fig. 2. Time course of production of cytogenin and its intermediates in cultured broth of *Streptoverticillium eurocidicum* MI43-37F11.

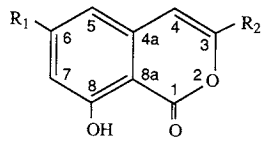
○ Cytogenin (**I**), ● (**II**), ⊡ (**III**), ■ (**IV**), + total isocoumarins.



methanol, suggesting the presence of the isocoumarin skeleton. The ^{1}H NMR spectrum of **III** revealed the absence of the OCH_3 group (δ_H 3.80) present in cytogenin. The EI-MS spectra of **III** and cytogenin showed a similar pattern with a mass shift of 14 dalton. These results together with ^{13}C NMR spectroscopic analysis indicated that **III** was 3-hydroxymethyl-6,8-dihydroxyisocoumarin as shown in Fig. 3. Structure of **IV** was ascribed to the replacement of the $-CH_2OH$ (δ_C 59.4, δ_H 4.30) present in cytogenin with $-CH_3$ (δ_C 18.8, δ_H 2.20) at C-3 position based on the ^{1}H, ^{13}C NMR and EI-MS spectroscopic analysis (*m/z*; 206 for **IV**, *m/z*; 222 for cytogenin). Structure of **II** was also ascribed to the replacement of the $-OCH_3$ (δ_C 55.8, δ_H 3.80) present in **IV** with $-OH$ (δ_H 10.8) at C-6 position by ^{1}H, ^{13}C NMR and EI-MS spectroscopic analysis. All assignments of ^{1}H and ^{13}C NMR signals for **II**, **III**, **IV** and cytogenin are summarized in Table 1. Structures of **II**, **III**, **IV** and cytogenin are shown in Fig. 3.

As shown in Fig. 2, compound **II** was produced at 20th hour cultivation at first. Thus, the identity of precursors was examined by adding ^{14}C-labeled acetate, propionate, mevalonate and formate to the culture at 20th hour cultivation. Since **I** and **IV** having OCH_3 group at the C-6 position were produced firstly at 30th hour cultivation, ^{14}C-labeled procursors for methyl group were added to the culture at 30th hour cultivation. As shown in Table 2, ^{14}C-acetate and methionine were efficiently incorporated into cytogenin. On the contrary, propionate, mevalonate and formate were not incorporated. These results suggest that the isocoumarin skeleton of cytogenin was derived from acetate and the methyl group of cytogenin from methionine. Then, the time course of incorporation of ^{14}C-acetate into cytogenin was investigated. As shown in Fig. 4, ^{14}C-acetate was efficiently incorporated into cytogenin from 20th to 30th hour cultivation. The total amounts of cytogenin and its analogs in the cultured broth were reached to maximum level within 30th hour cultivation (Fig. 2). These results indicate that the isocoumarin

Fig. 3. Structure of cytogenin (**I**) and its intermediates **II**, **III** and **IV**.



Cytogenin (**I**)	$R_1=OCH_3$	$R_2=CH_2OH$
3-Methyl-6,8-dihydroxyisocoumarin (**II**)	$R_1=OH$	$R_2=CH_3$
3-Hydroxymethyl-6,8-dihydroxyisocoumarin (**III**)	$R_1=OH$	$R_2=CH_2OH$
3-Methyl-6-methoxy-8-hydroxyisocoumarin (**IV**)	$R_1=OCH_3$	$R_2=CH_3$

Table 1. ^1H and ^{13}C NMR data of cytogenin, **II**, **III** and **IV** in DMSO-d_6.

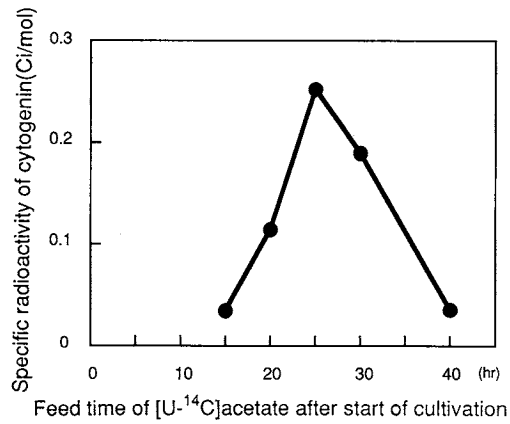
Compound	Cytogenin		**II**		**III**		**IV**	
Position	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H
1	165.1	—	165.4	—	166.1	—	165.3	—
3	157.4	—	154.0	—	156.9	—	154.4	—
4	102.9	6.67 (s)[a]	104.1	6.47 (d, ~1)	103.0	6.60 (s)	104.2	6.51 (d, ~1)
4a	139.1	—	139.6	—	139.2	—	139.5	—
5	101.8	6.68 (d, 2.0)	102.3	6.33 (d, 2.0)	103.3	6.43 (d, 2.0)	100.9	6.50 (d, 2.0)
6	166.5	—	165.6	—	165.2	—	166.4	—
7	100.6	6.54 (d, 2.0)	101.2	6.30 (d, 2.0)	101.8	6.32 (d, 2.0)	100.1	6.54 (d, 2.0)
8	162.6	—	162.6	—	162.7	—	162.5	—
8a	99.4	—	97.8	—	98.0	—	99.0	—
9	59.4	4.30 (d, 5.0)	18.7	2.22 (d, ~1)	59.5	4.25 (d, ~1)	18.8	2.20 (d, ~1)
6-OCH_3	55.9	3.80 (s)	—	—	—	—	55.8	3.80 (s)
6-OH	—	—	—	10.8 (br)	—	—	—	—
8-OH	—	10.9 (br)	—	10.9 (s)	—	10.9 (br)	—	11.0 (br)
9-OH	—	5.7 (t, 5.0)	—	—	—	5.7 (br)	—	—

Chemical shifts in ppm referenced to TMS at 0 ppm.
[a] Proton signal multiplicity and coupling constant (J=Hz).

Table 2. Incorporation of ^{14}C precursors into cytogenin.

Precursor	Specific activity (mCi/mol)	Incorporation ratio into cytogenin (%)
Sodium [1,2-$^{14}C_2$]acetate	880	3.18
Sodium [1-^{14}C]propionate	22	0.08
R-[2-^{14}C]mevalonic acid lactone	10	0.04
Sodium [^{14}C]formate	43	0.07
1-[*Methyl*-^{14}C]methionine	5,780	17.95

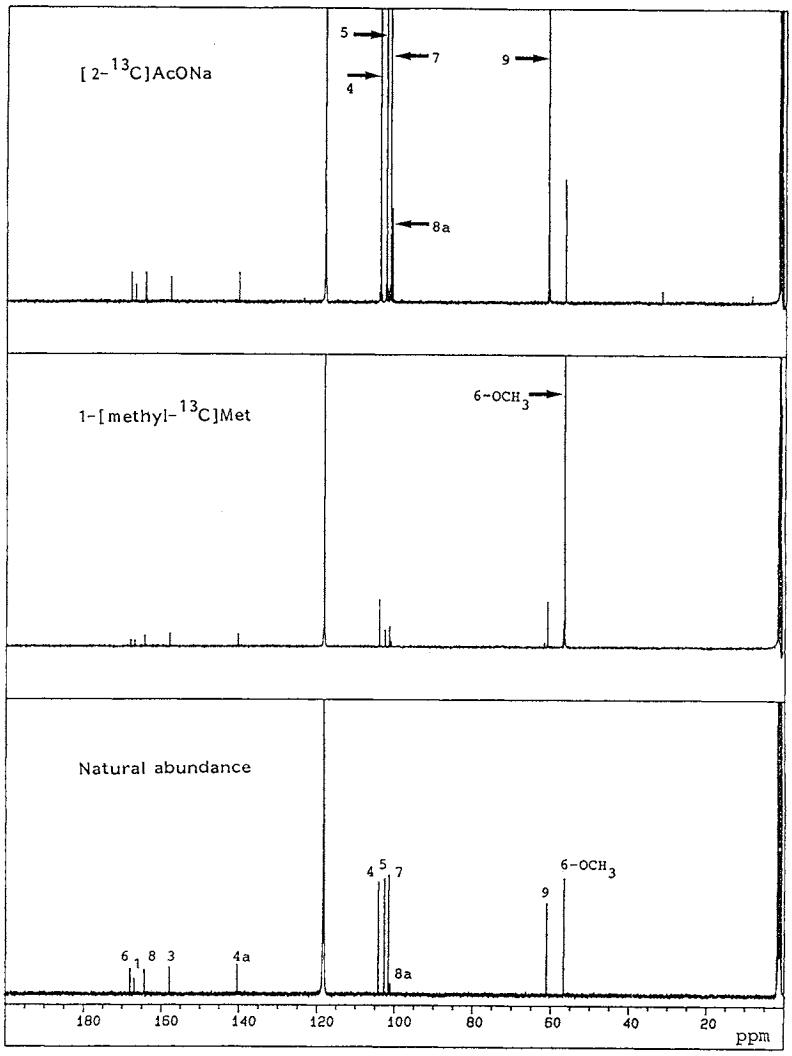
Fig. 4. Time course of [*U*-^{14}C]acetate incorporation into cytogenin.



skeleton was synthesized within 30th hour cultivation. Thus, the feeding time of ^{14}C-acetate is important and ^{14}C-acetate should be added within 20~30th hour after start of cultures to get cytogenin of high specific activity.

To determine the incorporation pattern of acetate and methyl group in cytogenin, feeding experiments using [2-^{13}C], [1,2-^{13}C]acetates and 1-[*methyl*-^{13}C]methionine were performed. The ^{13}C NMR spectrum

Fig. 5. ^{13}C NMR spectra of cytogenin derived from sodium [2-^{13}C]acetate and 1-[*methyl*-^{13}C]methionine (CD_3CN).



of cytogenin derived from [2-^{13}C]acetate as a precursor is shown in Fig. 5. Arrows indicate the enriched carbon signals. Enrichment ratios of carbon signals in [2-^{13}C]acetate-labeled cytogenin and ^{13}C-^{13}C coupling constants in [1,2-^{13}C]acetate-labeled cytogenin are listed in Table 3. Enrichment ratio of natural and [2-^{13}C]acetate-labeled cytogenin was expressed relative to the 6-OCH_3 signal as 1.0. Enrichment ratios were up to 3.63, 3.86, 4.35 and 4.82 for C-4, 5, 7 and 9, respectively. Although the signal of C-8a was not enriched against the signal of 6-OCH_3, it could be concluded that the C-8a was enriched, because the enrichment ratio of C-8a in [2-^{13}C]acetate-labeled cytogenin was about two times higher than that of C-8a in natural cytogenin. These results indicate that the isocoumarin skeleton of cytogenin is derived from five acetate units. The conclusion was verified by the enriched five pairs of ^{13}C-^{13}C coupling constants of the [1,2-^{13}C]acetate-labeled cytogenin (Table 3).

On the other hand, the ^{13}C NMR spectrum of cytogenin obtained by feeding of 1-[*methyl*-^{13}C]

Table 3. ^{13}C chemical shifts, enrichment ratio of cytogenin derived from ^{13}C-single-labeled precursors and the J_{C-C} of sodium [1,2-^{13}C]acetate labeled cytogenin.

Carbon	δ (ppm)	Enrichment ratio[c] of cytogenin derived from			[1,2-^{13}C]AcONa[a] J_{C-C} (Hz)
		Normal	[2-^{13}C]AcONa[a]	1-[*Me*-^{13}C]Met[b]	
1	166.9	0.18[c]	0.14[c]	0.11[d]	74
3	157.9	0.32	0.19	0.26	57
4	104.1	0.95	3.63	1.00	54
4a	140.4	0.29	0.23	0.39	54
5	103.6	0.77	3.86	0.37	68
6	168.0	0.30	0.21	0.14	68
7	101.5	1.07	4.35	0.38	72
8	164.3	0.28	0.23	0.24	72
8a	101.0	0.39	0.74	0.12	74
9	61.0	1.10	4.82	0.97	57
6-OCH_3	56.7	1.00	1.00	67.98	—

[a] AcONa: Sodium acetate.
[b] Met: Methionine.
[c,d] Enrichment ratios were relative to the intensity of 6-OCH_3 signal ([2-^{13}C]AcONa) as 1.0, and to the intensity of C-4 signal (1-[*Me*-^{13}C]Met) as 1.0, respectively.

methionine indicated the enhancement of carbon signal only in 6-OCH_3 as shown in Fig. 5 and Table 3. The arrow indicated the enriched carbon signal in the ^{13}C NMR spectrum of cytogenin produced by feeding 1-[*methyl*-^{13}C]methionine (Fig. 5). In Table 3, enrichment ratio of the carbon signals was calculated from the relative intensity of C-4 as 1.0. Enrichment ratio was up to 67.98 for 6-OCH_3.

From the results mentioned above, the isocoumarin skeleton of cytogenin is derived from a pentaketide intermediate obtained by head-to-tail condensation of five acetate units. The methyl group at 6-OCH_3 of cytogenin is derived from the methyl group of methionine. Cytogenin was produced by hydroxylation and/or methylation of intermediates (**II**, **III** and **IV**). Thus, the biosynthetic pathway of cytogenin (**I**) is suggested to be as follows; (A), **II**→**IV**→**I**; or (B), **II**→**III**→**I**. Pathway (A) is thought to be correct because the quantity of **IV** produced was apparently more than that of **III**. This proposed pathway of cytogenin biosynthesis is summarized in Fig. 6. The mode of formation of the isocoumarin skeleton and its methylation are similar to that of reticulol[2].

Finally, a high specific radioactive cytogenin (0.88 Ci/mol) was obtained by the method reported here.

Experimental

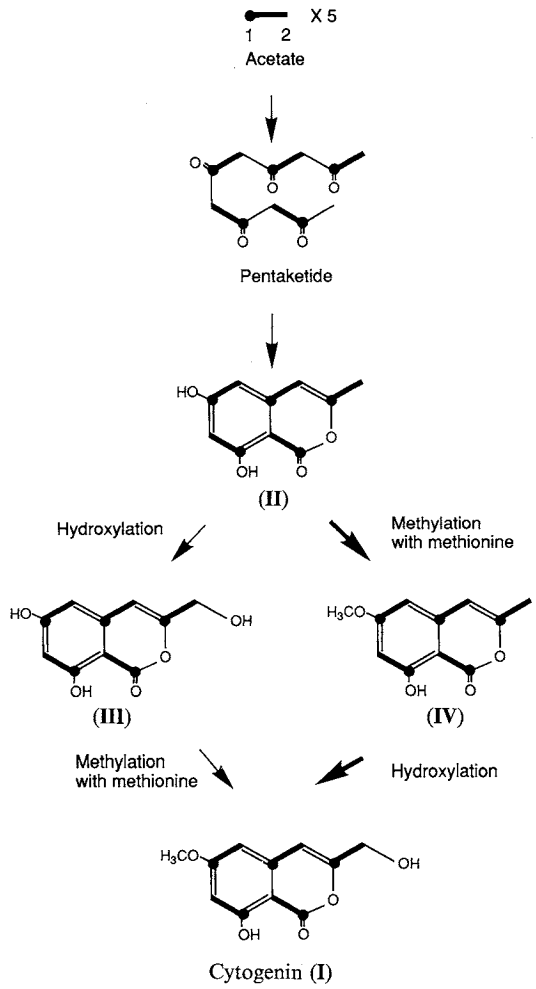
Labeled Compounds

1-[*Methyl*-^{14}C]methionine (53.4 mCi/mmol), sodium [1-^{14}C]propionate (57.0 mCi/mmol) were purchased from New England Nuclear, Boston, U.S.A. Sodium [*U*-^{14}C]acetate (52.0 mCi/mmol), *R*-[2-^{14}C]mevalonic acid lactone (53.9 mCi/mmol) and sodium [^{14}C]formate (51.0 mCi/mmol) were purchased from Amersham, Japan. Sodium [2-^{13}C]acetate (99% ^{13}C enriched), sodium[1,2-$C^{13}C$]acetate (99% ^{13}C enriched) and 1-[*methyl*-^{13}C]methionine (99% ^{13}C enriched) were purchased from Sigma, U.S.A.

Microorganism

Cytogenin-producing strain with high potency was obtained by single-spore isolation from *Streptoverticillium eurocidicum* MI43-37F11.

Fig. 6. Proposed pathway of cytogenin biosynthesis by *Streptoverticillium eurocidium* MI43-37F11.



Spectroscopy

UV and IR spectra were measured by a Hitachi 228A UV spectrophotometer and a Hitachi 228A IR spectrophotometer, respectively. ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR spectra in DMSO-d_6 were measured by a JEOL JNM-GX400 at room temperature. EI mass spectra (ionizing energy; 70 eV) were measured by a Hitachi PMU-6M mass spectrometer.

HPLC Analysis of Cytogenin and Its Intermediates in the Cultured Broth

At each time of cultivation, an aliquot of 2.0 ml of the cultured broth was collected and extracted with 2.0 ml of EtOAc. After centrifugation, 1.0 ml of EtOAc layer was evaporated and the residue was dissolved in 0.2 ml of CH_3CN. The concentration of cytogenin and its intermediates was determined by HPLC using an aliquot of 20 μl of the test samples. HPLC conditions were as follows: column, YMC-Pack A303 ODS (4.6 × 250 mm, Yamamura Chemical Laboratories Co., Ltd.); solvent system, 40% CH_3CN-H_2O; flow rate, 1.0 ml/minute; detection, UV at 244 nm. Cytogenin and its intermediates **II**, **III** and **IV** were eluted at 9.1, 11.0, 4.5 and 29.5 minutes of retention time, respectively.

Production of Cytogenin and Its Intermediates

A loopful of a spore suspension of *S. eurocidicum* MI43-37F11 grown on a yeast extract-soluble starch agar slant was inoculated into a 500-ml Erlenmeyer flask containing 110 ml of medium (glucose 2.0%, yeast extract 0.2%, soybean meal 1.5%, K_2HPO_4 0.1%, $MgSO_4 \cdot 7H_2O$ 0.005%, w/v, pH 6.5 before sterilization). The flask was incubated at 30°C for 2 days on a rotary shaker (180 rpm). The seed culture (2 ml) was transferred into a 500-ml Erlenmeyer flask containing 110 ml of production medium (glucose 5.0%, polypeptone 0.5%, yeast extract 0.05%, K_2HPO_4 0.1%, $MgSO_4 \cdot 7H_2O$ 0.005%, $CoCl_2 \cdot 6H_2O$ 0.002%, $FeSO_4 \cdot 7H_2O$ 0.0005%, $ZnSO_4 \cdot 7H_2O$ 0.0005%, $MnSO_4 \cdot 4H_2O$ 0.0005%, w/v, pH 6.8 before sterilization), and was cultured for 25, 30, 40 and 75 hours to produce **II**, **III**, **IV** and cytogenin, respectively.

Production of ^{13}C- or ^{14}C-labeled Cytogenin

To produce ^{13}C- or ^{14}C-labeled cytogenin, ^{13}C- or ^{14}C-labeled methionine was added to the flask at 30th hour cultivation. Other ^{13}C- or ^{14}C-labeled compounds were added to the flask at 20th hour cultivation. Fifty mg of ^{13}C-labeled compounds or 50 μCi of ^{14}C-labeled compounds dissolved in distilled water (0.5 ml) were added separately to the flasks. Then, the cultivation was continued for a total of 75 hours.

Preparation of Cytogenin

At 75th hour cultivation, the culture filtrate (3.0 liter) was extracted twice with EtOAc (1.5 liter). The organic layer was evaporated and purified by reverse phase HPLC. HPLC conditions were as follows; column, YMC-Pack A343 ODS (25 × 250 mm, Yamamura Chemical Laboratories Co., Ltd.); solvent

system, linear gradient of 20~80% CH_3CN-H_2O; flow rate, 5.0 ml/minute; detection, UV at 244 nm. The fraction containing cytogenin was loaded on a Sephadex LH-20 column, and was eluted with MeOH. The fraction containing cytogenin was concentrated *in vacuo* to afford 4.5 mg of white powder: UV_{max}^{MeOH} nm (log ε) 238 (4.63), 244 (4.65), 256 (sh, 4.05), 274 (sh, 3.82), 286 (sh, 3.67), 330 (3.78); IR (KBr) 3480, 1680, 1630, 1230, 1190 cm^{-1}; EI-MS *m/z* 222 (M^+); ^1H and ^{13}C NMR data are listed in Table 1.

Preparation of **II**

At 25th hour cultivation, the culture filtrate (3.2 liters) was extracted twice with EtOAc (1.6 liters). **II** was purified by the same methods used for the preparation of cytogenin to afford 3.5 mg of white powder: EI-MS *m/z* 192 (M^+); IR (KBr) 3250, 1675, 1625, 1180 cm^{-1}; ^1H and ^{13}C NMR data are listed in Table 1.

Preparation of **III**

At 30th hour cultivation, the culture filtrate (3.0 liters) was extracted twice with EtOAc (1.5 liters). **III** was purified by the same methods used for the preparation of cytogenin to afford 3.0 mg of white powder: EI-MS *m/z* 208 (M^+); IR (KBr) 3350, 1685, 1620, 1240, 1170 cm^{-1}; ^1H and ^{13}C NMR data are listed in Table 1.

Preparation of **IV**

At 40th hour cultivation, the culture filtrate (3.0 liters) was extracted twice with EtOAc (1.6 liters). **IV** was purified by the same methods used for the preparation of cytogenin to afford 5.0 mg of white powder: EI-MS *m/z* 206 (M^+); IR (KBr) 1680, 1640, 1235, 1160 cm^{-1}; ^1H and ^{13}C NMR data are listed in Table 1.

Incorporation of ^{14}C-labeled Compounds into Cytogenin

At 75th hour cultivation, the culture filtrate (200 ml) was extracted twice with EtOAc (100 ml). The organic layer was evaporated to dryness and the residue was dissolved in CH_3CN (1.0 ml). An aliquot of 5 μl of the solution was analyzed by HPLC in the same condition as that of analysis of natural cytogenin. To measure the specific radioactivity of ^{14}C-cytogenin, the ^{14}C-cytogenin fraction, purified by HPLC, was measured in a scintillation spectrometer (LSC-700, Aloka, Japan). Specific radioactivity of ^{14}C-labeled cytogenin is shown in Table 2.

Preparation of ^{13}C-labeled Cytogenin

^{13}C-labeled cytogenin was isolated by the method reported previously[1]. Briefly, the culture filtrate (520 ml) was extracted twice with EtOAc (260 ml) and purified by silica gel column chromatography ($CHCl_3$-MeOH 50:1), reverse phase HPLC (45% CH_3CN-H_2O) and Sephadex LH-20 column chromatography (MeOH). ^{13}C NMR data of ^{13}C-labeled cytogenin are listed in Table 3.

Acknowledgments

This work was supported in part by a Grant-Aid for Cancer Research from the Ministry of Education, Science and Culture, Japan.

References

1) KUMAGAI, H.; T. MASUDA, M. OHSONO, S. HATTORI, H. NAGANAWA, T. SAWA, M. HAMADA, M. ISHIZUKA & T. TAKEUCHI: Cytogenin, a novel antitumor substance. J. Antibiotics 43: 1505~1507, 1990

2) FURUTANI, Y.; I. TSUCHIYA, H. NAGANAWA, T. TAKEUCHI & H. UMEZAWA: Biosynthetic studies of reticulol, an isocoumarin, by ^{13}C NMR spectroscopy. Agric. Biol. Chem. 41: 1581~1585, 1977