Available online at www.sciencedirect.com
SCIENCE DIRECT®
European Journal of Medicinal Chemistry 40 (2005) 662–673
EUROPEAN JOURNAL OF MEDICINAL CHEMISTRY
www.elsevier.com/locate/ejmech

Preparations of vitamin D analogs, spirostanols and furostanols from diosgenin and their cytotoxic activities

Hong-Ji Quan [b], Jyunichi Koyanagi [a], Fusao Komada [a], Setsuo Saito [a,*]

[a] *Faculty of Pharmaceutical Sciences, Josai University, Keyakidai 1-1, Sakado, Saitama 350-0295, Japan*
[b] *College of Pharmacy, Yanbian University, Yanji, Jilin 133000, China*

Received 29 October 2004; revised and accepted 3 February 2005

Available online 07 April 2005

Abstract

Vitamin D analogs **12** and **13** having a spiro ring in the side chain, various spirostanols **18**–**21**, **26**, **27**, **29** and **37**, and furostanols **34**–**36** having SCN and SeCN groups at the 26 position were prepared from diosgenin **1** via (20*S*,22*R*,25*R*)-spirost-1α,2α-epoxy-4,6-dien-3-one **19** as a key intermediate. The cytotoxic activities of these derivatives as well as **1** on scarcely P-gp-expressed HCT 116 cells and P-gp-overexpressed Hep G2 cells were examined by MTT assay. Furostanols **34** (IC_{50} value: 4.9 ± 0.3 μM) and **36** (IC_{50} value: 1.3 ± 0.2 μM) exhibited marked cytotoxic effects on HCT 116 cells, and spirostanol **29** (IC_{50} value: 2.4 ± 0.8 μM) and furostanol **36** (IC_{50} value: 2.8 ± 0.4 μM) on Hep G2 cells. Furthermore, the effects of vitamin D analog **12**, spirostanol **26** and furostanol **36** on apoptosis-signaling pathways were investigated. Compounds **12** and **26** overexpressed *p53* and *Bax* mRNAs, while compound **36** overexpressed only *Bax* mRNA.

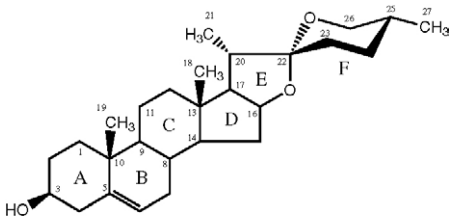
Keywords: Vitamin D analog; Spirostanol; Furostanol; Cytotoxicity; Apoptosis

1. Introduction

The overexpression of the P-glycoprotein (P-gp) which causes multidrug resistance (MDR 1) to some cytotoxic compounds in tumor cells has been a significant obstacle for successful chemotherapy of many cancers [1–3]. Recently, a number of modulators have been studied in detail to overcome these obstacles [4,5]. These modulators included calcium channel blockers, calmodulin antagonists, steroidal agents, protein kinase C inhibitors, immunosuppressive drugs and antibiotics as well as surfactants [5]. However, these agents often give disappointing results in vivo because their low binding affinities require the use of high doses, resulting in unacceptable toxicity [4]. Today, the aim is to obtain useful modulators and potent cytotoxic compounds which are not influenced by efflux systems such as P-gp. We have attempted to obtain compounds that are highly toxic to the cancer cells which overexpress P-gp.

Diosgenin (**1**) is an aglycon of dioscin isolated from rhizomes of *Dioscorea cokoro* Makino (*Dioscoreaceae*) [6] and rhizomes of *Trillium erectum* Linne (*Liliaceae*) [7]. Compound **1** has a hydroxyl group and an olefin bond at the C-3 and C-5 positions, respectively. The E and F rings which are a tetrahydrofuran ring and a tetrahydropyran ring, respectively, of **1** were fused at the C-22 position to form a spiro ring (Fig. 1). These functional groups, bonds and rings are available for the structural conversion of **1**, and some of the resulting products will be useful as pharmacologically active compounds or precursors for their synthesis (Fig. 1).

Previously [8] we reported the syntheses of diosgenin derivatives, (20*S*,22*S*,25*R*)-22-thiospirost-5-en-3β-ol **2**, (20*S*,22*S*,25*R*)-22-selenospirost-5-en-3β-ol **3** and solasodine **4**, having hetero atoms such as sulfur (S), selenium (Se) and



1

Fig. 1. Structure of diosgenin **1**.

* Corresponding author. Tel.: +81 49 271 7667; fax: +81 49 271 7984.
E-mail address: saitoset@josai.ac.jp (S. Saito).

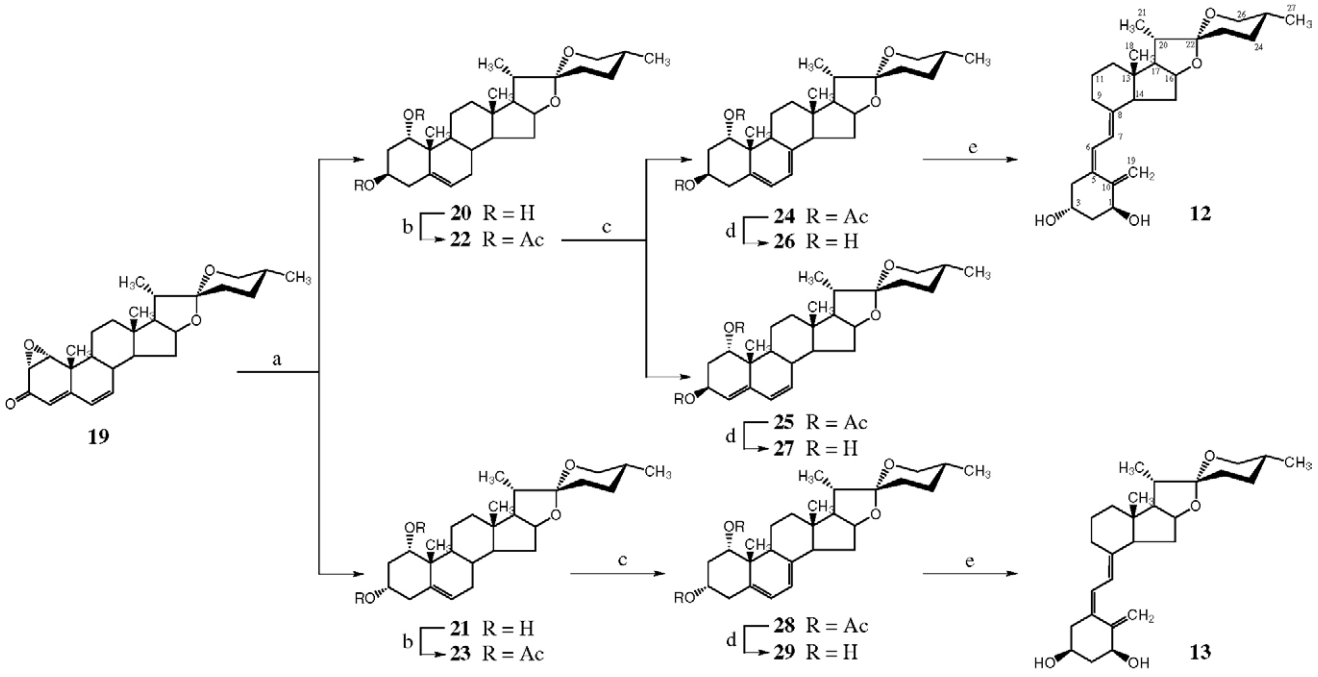
0223-5234/$ - see front matter © 2005 Elsevier SAS. All rights reserved.
doi:10.1016/j.ejmech.2005.02.003

nitrogen (as NH), respectively, in the F ring, α,β-unsaturated derivatives, (20*S*,22*S*,25*R*)-22-thiospirost-4-en-3-one **5**, (20*S*,22*S*,25*R*)-22-selenospirost-4-en-3-one **6**, solasodinone **7** and diosgenone **8** having S, Se, NH and O, respectively, in the F ring, dienone derivatives, (20*S*,22*R*,25*R*)-22-spirost-1,4-dien-3-one **9** and (20*S*,22*S*,25*R*)-22-thiospirost-1,4-dien-3-one **10**, having O and S, respectively, in the F ring, and 26-cyanoselenopseudodiosgenone **11** from **1**. We have also compared their pharmacological effects on the inhibition of interferon-γ production, cytotoxic activities, antiurease activities and antibacterial activities with those of **1**. The present study deals with preparations of vitamin D analogs having a spiro ring and various spirostanols and furostanols containing several functional groups in the molecules from **1**. Furthermore, the cytotoxic activities of the synthesized compounds are evaluated using human colorectal (HCT 116) and human hepatoma (Hep G2) cancer cell lines, and also the apoptosis-signaling pathways activated by three different types of compounds, a vitamin D analog **12**, a spirostanol **26**, and a furostanol **36**, in Hep G2 cell lines are investigated using *p53*, *p21*, *Bax* and *Bcl-2* mRNAs [9].

2. Chemical results and discussion

Our initial purpose was to obtain vitamin D type derivatives **12** and **13** having a spiro ring at the side chain from **1** and to evaluate the cytotoxic activities of the derivatives (Scheme 1). Many papers devoted to the synthesis of vitamin D analogs have been published [10–17]. In these syntheses, cholesterol **14** and 24-oxocholesterol **15** having a double bond at the five position and provitamin D **16** having a *cis* diene structure in the B ring were utilized as starting materials (Fig. 2). As the structure of the A ring of diosgenin **1** is the same as that of **14** and **15**, **1** was used as a starting material for the synthesis of vitamin D analogs having a spiro ring at the side chain (Scheme 1 and Fig. 2).

Liu et al. [18] reported the synthesis of (25*R*)-ruscogenin (**17**) which is the aglycon of (25*R*)-ruscogenin-1-yl-β-D-xylopyranosyl-(1 → 3)-[β-D-glucopyranosyl-(1 → 2)]-β-D-fucopyranoside obtained from the tuber of *Liriope muscari* (*Liliaceae*). Compound **17** was prepared from diosgenin **1** as a starting material. In the synthesis of **17**, epoxide **19** was obtained from **1** via (20*S*,22*R*,25*R*)-spirost-1,4,6-trien-3-one **18**. Then **19** was reacted with Li/NH_3 in THF, followed by treatment with NH_4Cl, to obtain (20*S*,22*R*,25*R*)-spirost-1α,3β-dihydroxy-5-ene (**20**) which was a key intermediate for the synthesis of **17**, together with (20*S*,22*R*,25*R*)-spirost-1α,3β-dihydroxy-6-ene and unidentified products. As compound **20** was also thought to be an intermediate of vitamin D analog **12**, which was obtained by the minor modification of the procedure reported by Liu et al. [18]; Birch reduction of **19** with Na in liquid NH_3 instead of Li in liquid NH_3 gave two products, **20** and its epimer **21** with respect to the three position in yields of 44.1% and 5.9%, respectively. The ^{1}H-NMR spectrum of **20** was consistent with that reported by Liu et al. Compound **21** showed the same quasimolecular ion peak as that of **20** at *m/z* 431 $[M + H]^+$ in the fast atom bombardment mass spectrum (FABMS). The ^{13}C-NMR spectrum of **21** was superimposable with that of **20** (Table 1). The configuration at the three position was confirmed by comparison of the ^{1}H-NMR spectrum of **21** with that of **20**; while the signal of H-3α of **20** was observed as a multiplet at δ 3.98, the signal of H-3β of **21** was exhibited as a broad singlet at δ 4.14. These



Scheme 1. Preparations of vitamin D analogs **12** and **13**. Reagents and conditions: (a) i) Na/NH_3, THF, -60 °C, ii) NH_4Cl; (b) Ac_2O, pyridine; (c) i) NBS, *n*-hexane, reflux, 2 h, ii) γ-collidine, xylene, reflux 2 h; (d) KOH, MeOH, benzene, 65 °C, 1 h; (e) i) Irradiation, Et_2O, 0 °C, 2 h, ii) Et_2OH, reflux, 2 h.

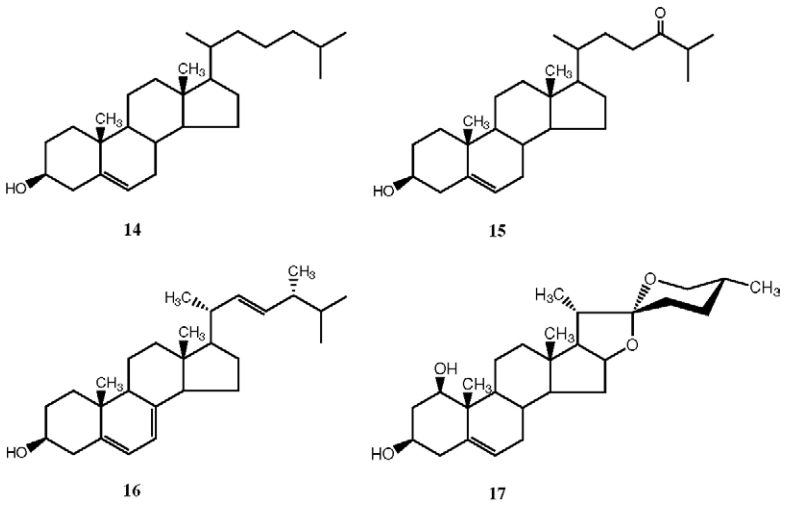


Fig. 2. Structures of compounds **14**–**17**.

spectral data suggest that **21** is (20*S*,22*R*,25*R*)-spirost-1α,3α-dihydroxy-5-ene.

1α,3β-Diacetate **22** derived from **20** by acetylation was reacted with *N*-bromosuccinimide (NBS), followed, without further purification of the resulting mixture, by treatment with γ-collidine to give two products, (20*S*,22*R*,25*R*)-spirost-1α,3β-diacetoxy-5,7-diene **24** and (20*S*,22*R*,25*R*)-spirost-1α,3β-diacetoxy-4,6-diene **25** in yields of 53.0% and 4.8%, respectively. Both products showed the same ion peak at *m*/*z* 513 $[M + H]^+$ in the FABMS. The ^{13}C-NMR spectrum of **24** showed four vinyl carbon signals at δ 139.9, 135.2, 121.5 and 115.6 due to C-8, C-5, C-6 and C-7, respectively. Two vinyl proton signals were observed at δ 5.67 (dd, J = 6.1 and 2.7 Hz) and 5.39 (quintet, J = 2.7 Hz) due to H-6 and H-7, respectively, in the ^{1}H-NMR spectrum of **24**. Compound **25** also showed four vinyl carbon signals at δ 142.5, 131.5, 127.9 and 121.1 assignable to C-5, C-7, C-6 and C-4, respectively, in the ^{13}C-NMR spectrum. The ^{1}H-NMR spectrum of **25** showed three vinyl proton signals at δ 5.98 (dd, J = 9.8 and 2.8 Hz), 5.68 (dd, J = 9.8 and 1.5 Hz) and 5.42 (broad s) due to H-6, H-7 and H-4, respectively. Alkaline hydrolysis of **24** and **25** gave compounds **26** and **27** in yields of 92.9% and 75.8%, respectively.

The successive irradiation and isomerization [10,19] of 5,7-diene **26** gave the desired vitamin D derivative **12** in 20.0% yield, which showed an ion peak at *m*/*z* 429 $[M + H]^+$ in the FABMS. In the ^{13}C-NMR spectrum of **12**, six vinyl carbon signals were observed at δ 147.5, 141.8, 133.6, 124.5, 117.7 and 111.8 assignable to C-10, C-8, C-5, C-6, C-7 and C-19, respectively. The ^{1}H-NMR spectrum of **12** exhibited four vinyl proton signals at δ 6.34 (d, J = 11.3 Hz), 6.00 (d, J = 11.3 Hz), 5.29 (d, J = 1.8 Hz) and 4.97 (d, J = 1.8 Hz) due to H-6, H-7, H-19a and H-19b, respectively (Table 1).

Successive bromination and dehydrobromination of diacetate **23** derived from **21** was performed according to the method for the preparation of **24** to obtain (20*S*,22*R*,25*R*)-spirost-1α,3α-diacetoxy-5,7-diene **28** in 25.1% yield. The FABMS of **28** showed the same ion peak at *m*/*z* 513 $[M + H]^+$ as that of **24**. The ^{13}C-NMR spectrum of **28** was superimposable with that of **24** (see Section 5.1.1.4.). The successive irradiation and isomerization of **29** obtained from **28** gave another vitamin D derivative **13** in 20.0% yield, which showed the same ion peak at *m*/*z* 429 $[M + H]^+$ as **12** in the FABMS. Also the ^{13}C-NMR spectrum of **13** was superimposable with that of **12**.

As we succeeded in obtaining vitamin D type derivatives **12** and **13** having a spiro ring at the side chain, we next attempted to synthesize their analogs, such as (20*S*,22*S*,25*R*)-9,10-secothiospirosta-5,7,10(19)-trien-1α,3β-diol **40** and (20*S*,22*S*,25*R*)-9,10-secoselenospirosta-5,7,10(19)-trien-1α,3β-diol **41**, having a sulfur or a selenium atom instead of an oxygen atom in the heterospiro ring. Compound **24** was heated in acetic anhydride to give triacetate **30** in 78.3% yield, which showed an ion peak $[M + H]^+$ at *m*/*z* 555. The triacetate was hydrolyzed with methanolic KOH at room temperature to give crude triol **31** which showed an ion peak $[M + H]^+$ at *m*/*z* 429 (Fig. 3).

With respect to *p*-toluenesulfonylation (tosylation) of an alcohol, Yoshida et al. [20] recently reported that i) tosylation of some alcohols with *p*-toluenesulfonyl chloride (TsCl) using pyridine as a catalyst and solvent gave undesirable products, ii) tosylation using a catalyst of $Et_3N/Me_3N{\cdot}HCl$ (ca. 20:1) in CH_2Cl_2 proceeded faster than the case of i) and gave the desired tosylates. Here we performed both tosylations. The triol **31** reacted with TsCl using $Et_3N/Me_3N{\cdot}HCl$ (ca. 20:1) in CH_2Cl_2 to give tosylate **32** in a yield of 69.4%. The FABMS spectrum of **32** showed an peak $[M + H]^+$ at *m*/*z* 583. Compound **32** was reacted with sodium iodide to give crude compound **33** (with a molecular ion peak $[M]^+$ at *m*/*z* 538). Compound **33** was reacted, without further purification, with KSCN in DMF at 70 °C to give 26-thiocyanate **34** (38.1% yield from **32**) which showed a molecular ion peak $[M]^+$ at *m*/*z* 469 in the FABMS spectrum. Similarly, **33** was reacted with KSeCN to give 26-selonocyanate **35** (24% yield from **32**). The FABMS spectrum of **35** showed a molecular ion peak $[M]^+$ at *m*/*z* 517 (Fig. 3).

Table 1
^{13}C-NMR spectral data of compounds provided for undergoing bioassay [a]

	18	19	20	21	26	27	29
C-1	152.8 [b]	59.4	72.8	73.2	72.7	72.0	72.4
C-2	128.1	54.7	38.3	33.4	38.5	35.5	33.0
C-3	186.2	194.6	66.3	68.4	65.3	64.7	67.7
C-4	123.8	119.6	41.4	39.8	40.0	125.4	38.4
C-5	162.4	158.5	137.4	135.3	136.3	140.8	134.0
C-6	127.7	127.9	125.3	126.3	121.9	128.6	122.9
C-7	138.0	139.9	31.9	32.0	115.4	131.1	115.0
C-8	37.7	37.1	31.4	31.3	140.0	36.7	141.0
C-9	48.4	46.1	41.6	41.6	37.6	44.4	37.4
C-10	41.2	38.9	41.8	42.9	42.4	39.4	43.5
C-11	21.7	21.0	20.1	19.8	20.7	20.2	20.0
C-12	39.4	39.3	39.5	39.5	39.1	39.6	39.0
C-13	41.0	41.0	40.2	40.2	41.1	41.5	40.8
C-14	53.3	53.3	56.4	56.4	54.5	53.8	54.4
C-15	31.2	31.2	31.8	31.9	30.9	31.4	30.8
C-16	80.4	80.3	80.8	80.8	80.6	80.6	80.7
C-17	61.9	61.9	62.0	62.0	61.9	62.0	61.6
C-18	16.3	16.2	16.3	16.3	16.4	16.3	16.3
C-19	20.8	18.5	19.5	19.2	16.5	19.5	16.5
C-20	41.6	41.6	41.6	41.4	42.1	41.4	42.0
C-21	14.5	14.5	14.5	14.5	14.5	14.4	14.3
C-22	109.3	109.3	109.3	109.3	109.3	109.2	109.4
C-23	31.3	31.3	31.4	31.4	31.3	31.3	31.2
C-24	28.8	28.8	28.8	28.8	28.8	28.7	28.7
C-25	30.2	30.2	30.3	30.3	30.3	30.2	30.2
C-26	66.9	66.9	66.8	66.8	66.9	66.8	66.7
C-27	17.1	17.1	17.1	17.2	17.1	17.1	17.1
	12	**13**	**34**	**35**	**36**	**37**	
C-1	70.7	73.3	72.7	72.7	72.4	72.5	
C-2	42.7	40.5	38.4	38.4	38.4	38.4	
C-3	66.8	68.2	65.3	65.2	66.8	66.8	
C-4	45.2	45.6	39.9	39.9	36.4	36.4	
C-5	133.6	132.3	136.4	136.4	37.0	37.1	
C-6	124.5	125.2	121.8	121.7	130.5	130.3	
C-7	117.7	117.7	115.6	115.6	124.6	124.7	
C-8	141.8	141.9	139.5	139.5	126.2	125.9	
C-9	28.4	28.5	37.6	37.6	41.9	42.0	
C-10	147.5	147.2	42.3	42.3	39.8	39.7	
C-11	23.4	23.4	20.8	20.8	19.1	19.1	
C-12	40.2	40.3	38.9	38.9	36.4	36.9	
C-13	43.8	43.9	43.8	43.8	43.5	40.5	
C-14	56.4	56.4	52.8	52.8	144.6	145.7	
C-15	30.7	30.8	32.5	33.1	34.5	33.0	
C-16	80.3	80.4	84.1	84.1	83.8	79.6	
C-17	62.4	62.5	63.9	63.9	64.0	61.3	
C-18	16.3	16.4	14.0	14.0	22.2	24.1	
C-19	111.8	112.8	16.4	16.4	12.3	12.3	
C-20	42.1	42.1	104.5	104.5	104.6	43.1	
C-21	14.4	14.5	11.5	11.5	11.8	14.2	
C-22	109.3	109.3	150.9	150.9	151.0	109.0	
C-23	31.3	31.4	33.1	33.1	32.5	31.4	
C-24	28.7	28.8	23.0	23.1	23.0	28.7	
C-25	30.2	30.3	33.2	33.6	33.2	30.3	
C-26	66.8	66.9	41.2	37.6	41.2	67.0	
C-27	17.1	17.2	18.4	19.2	18.3	17.1	
CN	–	–	112.8	102.0	112.7	–	

[a] Spectra were obtained in $CDCl_3$.
[b] Chemical shifts were in ppm from internal $(CH_3)_4Si$. Signal assignments were based on DEPT, ^{1}H–^{1}H, ^{1}H–^{13}C COSY and ^{1}H–^{13}C-long-range COSY spectral data.

When the tosylation of **31** derived from **30** with TsCl was performed in pyridine solution instead of CH_2Cl_2 solution containing $Et_3N/Me_3N{\cdot}HCl$ (ca. 20:1), followed by successive reactions with NaI and KSCN, without any purification at each step because of unisolation of the mixture at each step, compounds **36** (*m/z* 469 $[M + H]^+$, colorless foam) and **37** (*m/z* 429 $[M]^+$, m.p. 201–203 °C) were obtained in 9.7% and 12.6% yields, respectively, from **30**. In the ^{13}C-NMR spectrum, **36** showed six vinyl carbon signals; C-22 and C-20 on the E ring at δ 151.0 and 104.6, respectively, and C-14, C-6, C-8 and C-7 at δ 144.6, 130.5, 126.2 and 124.6, respectively. Furthermore **36** showed the carbon signal of the SCN group at δ 112.7. Compound **37** showed the carbon signal of C-22 on the spiro ring at δ 109.0, and four vinyl carbon signals at δ 145.7, 130.3, 125.9 and 124.7 due to C-14, C-6, C-8 and C-7, respectively, by HMBC. In the successive reactions from **31** to **36** and **37**, the 5,7-*cis* diene changed to the 6,8(14)-*trans* diene. This rearrangement may probably have occurred during the tosylation of **31** using pyridine as catalyst and solvent.

Compound **32** was treated with 3 M HCl–methanol [21] to give compound **38** (48.9% yields), which showed an ion peak $[M + H]^+$ at *m/z* 583 in the FABMS and six vinyl carbon signals at 151.3, 144.6, 130.5, 126.2, 124.6 and 104.2 due to C-22, C-14, C-6, C-8, C-7 and C-20, respectively, in the ^{13}C-NMR spectrum. Thus, as the rearrangement from the 5,7-*cis* diene of **32** to the 6,8(14)-*trans* diene of **38** occurred easily in 3 M HCl–methanol, it is thought that the rearrangement of the *cis* diene of **31** in the tosylation with tosyl chloride in pyridine occurred by the action of *p*-toluenesulfonic acid generated in the tosylation. Reaction of **38** with NaI, followed by treatment with KSCN, gave compound **36** in a yield of 75.8%.

Irradiation of compounds **34** and **35** to obtain vitamin D analogs having hetero atoms, such as sulfur and selenium atoms instead of an oxygen atom, in the side ring failed because of the formation of mixtures that could not be isolated.

3. Pharmacological results and discussion

The cytotoxic activities of synthetic vitamin D derivatives (**12** and **13**), spirostanol derivatives (**18**–**21**, **26**, **27**, **29** and **37**) and furostanol derivatives (**34**–**36**) obtained in this study were compared with that of **1** using human colorectal (HCT 116) and human hepatoma (Hep G2) cancer cell lines. It is known that while the former cell lines scarcely express MDR 1 (P-glycoprotein; P-gp) [22], the latter cell lines overexpress it [23]. P-gp acts as an efflux pump to remove several antitumor agents, Ca^{2+} antagonists, cyclosporine, digoxin and other compounds from cells [24]. The cytotoxic activities of the compounds were tested by MTT assay [25], and the IC_{50} values were calculated based on the percentage inhibition of cell growth and are listed in Table 2.

Table 2 shows that in the cases of both HCT 116 and Hep G2 cells, the IC_{50} values of compounds **12**, **13**, **18**–**21**, **26**,

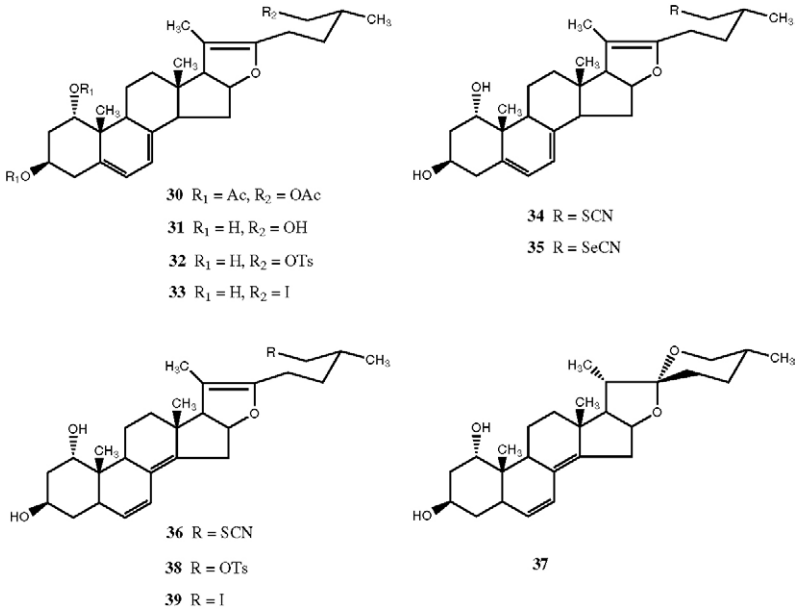


Fig. 3. Structures of compounds **30**–**39**.

27, **29** and **34**–**36** were lower than those of diosgenin **1** (92.3 ± 18.2 on HCT 116 and 21.0 ± 7.5 μM on Hep G2), indicating that the cytotoxic effects of the synthetic compounds increased compared with **1**.

In the case of HCT 116, the furostanol derivatives **34** and **36** more marked effects (IC_{50} values: 4.9 ± 0.3 for **34** and 1.3 ± 0.2 μM for **36**) than vitamin D derivatives **12** and **13** (IC_{50} values: 22.4 ± 5.1 and 16.9 ± 0.3 μM, respectively) and spirostanol derivatives **18**–**21**, **26**, **27**, **29** and **37** (IC_{50} values: 9.5 ± 2.6–54.0 ± 3.6 μM). Furostanol derivatives **34** and **35** had a 5,7-*cis* diene group and were substituted with a SCN group and a SeCN group, respectively, at the 26 position. Furostanol derivative **36** had a 6,8(14)-*trans* diene group and was substituted with a SCN group at the 26 position. These differences in structures and substituents might influence the appearance of a potent inhibitory effect on the growth of HCT 116 cells. The *trans* diene compound **36** having a SCN group at the 26 position had the most potent cytotoxic effect of all synthetic compounds on HCT 116 cells.

Table 2
Cytotoxic effects of **1**, **12**, **13**, **18**–**21**, **26**, **27**, **29** and **34**–**37** on HCT 116 and Hep G2 cells

	IC_{50} (μM)[a]	
Compound	HCT116	Hep G2
1 [S][b]	92.3 ± 18.2	21.0 ± 7.5
12 [V]	22.4 ± 5.1	16.0 ± 2.1
13 [V]	16.9 ± 0.3	16.3 ± 2.9
18 [S]	16.0 ± 0.4	10.3 ± 1.8
19 [S]	12.3 ± 0.5	10.3 ± 2.8
20 [S]	10.5 ± 0.5	5.2 ± 1.4
21 [S]	15.1 ± 0.4	5.9 ± 0.6
26 [S]	10.4 ± 0.3	6.8 ± 0.5
27 [S]	54.0 ± 3.6	7.7 ± 1.5
29 [S]	9.5 ± 2.6	2.4 ± 0.8
34 [F]	4.9 ± 0.3	6.4 ± 1.2
35 [F]	10.4 ± 1.0	12.7 ± 2.2
36 [F]	1.3 ± 0.2	2.8 ± 0.4
37 [S]	22.7 ± 0.6	21.6 ± 4.7

[a] IC_{50} values (mean ± S.D.) are the concentrations at which 50% of the cells are inhibited from growing.

[b] [S], [F] and [V] represent spirostanol, furostanol and vitamin D derivative, respectively.

In the case of Hep G2 cells, no structural relationship such as that shown in the case of HCT 116 cells was observed. In this case, not only furostanol derivative **36** but also spirostanol derivative **29**, which had a 5,7-*cis*-diene group, showed similar potent cytotoxic activities (2.8 ± 0.4 and 2.4 ± 0.8 μM, respectively).

Interestingly, the cytotoxic activities of all compounds synthesized in this study on Hep G2 cells were more potent than those on HCT 116 cells (Table 2) except for **34**–**36**, although **34** and **36** showed potent activities on HCT 116 and Hep G2 cells. Especially spirostanol derivative **29** and furostanol derivative **36** had marked cytotoxic activities (2.4 ± 0.8 and 2.8 ± 0.4 μM, respectively) among the tested compounds even on Hep G2 cells which overexpress P-gp. Furthermore, spirostanol derivatives **20**, **21**, **26** and **27** and furostanol derivative **34** were very cytotoxic (5.2 ± 1.4–7.7 ± 1.5 μM) to Hep G2 cells, although these derivatives were less effective than **29** and **36**. These results indicate that spirostanol derivatives **20**, **21**, **26** and **27** and furostanol derivatives **34** and **36** as well as **29** may be less sensitive substrates for the P-gp transport of Hep G2 cells as far as cytotoxic activity is concerned.

Here, we attempted to clarify the intracellular target of the compounds and the apoptosis-signaling pathway activated by these compounds in Hep G2 cell lines. For this investigation, different structural types of compounds **12**, **26** and **36** from vitamin D, spirostanol and furostanol derivatives, respectively, were selected and investigated for their effects on the expression of *p53*, *Bax*, *p21* and *Bcl-2* mRNAs. The results are listed in Table 3.

Table 3
Effect of derivatives **12**, **26** and **36** on apoptosis-related mRNA expression signals in Hep G2 cells

mRNA	12	26	36
p53	1.7 ± 0.5	2.2 ± 0.1	1.1 ± 0.2
Bax	2.5 ± 0.3	2.1 ± 0.4	2.4 ± 0.3
p21	0.9 ± 0.1	0.8 ± 0.1	0.9 ± 0.2
Bcl-2	0.7 ± 0.3	0.6 ± 0.3	1.0 ± 0.1

Values showed the ratios for the expression levels of these apoptosis-related mRNA signals with drug treatment/the expression levels of control (DMSO only). $n = 3$.

Table 3 shows that **12** and **26** produced significant increases in the expression levels of apoptosis-related signals such as *p53* mRNA (1.7-fold for **12** and 2.2-fold for **26**) and *Bax* mRNA (2.5-fold for **12** and 2.1-fold for **26**), while the expression levels of *p21* mRNA (0.9-fold for **12** and 0.8-fold for **26**) and *Bcl-2* mRNA (0.7-fold for **12** and 0.6-fold for **26**) were unchanged, indicating that **12**- and **26**-induced apoptosis in Hep G2 cells was mediated via the activation of both *p53* and *Bax*. In contrast, furostanol type derivative **36** overexpressed only *Bax* mRNA (2.4-fold) but not the other three mRNAs (0.9-fold–1.1-fold).

4. Conclusions

Vitamin D type derivatives having the spiro ring and various spirostanol and furostanol derivatives were synthesized from **1**. Successive oxidation and epoxidation of **1** gave an important intermediate **19** which was converted to diols **20** and **21** by Birch reduction. Diacetate **22** derived from **20** was reacted with NBS to give 1α,3β-diacetoxy-5,7-*cis*-diene derivative **24** and 1α,3β-diacetoxy-4,6-*trans*-diene derivative **25** in 50.3% and 4.8% yields, respectively. The former diene was deacetylated to give **26** which was successively irradiated to obtain the desired vitamin D derivative **12** in 20.0% yield. Similarly 1α,3α-diacetoxy-5,7-*cis*-diene derivative **28** was obtained from diacetate **23** derived from **21**. Compound **28** was deacetylated to give diol **29** which was irradiated to obtain vitamin D derivative **13** in 20.0% yield.

Next the synthesis of vitamin D derivatives having heterospiro rings such as **40** and **41** was attempted. Acetolysis of compound **24** gave triacetate **30** which was successively deacetylated, tosylated with $Et_3N/Me_3N{\cdot}HCl$ in CH_2Cl_2 and iodinated to obtain iodide **33**. Reaction of **33** with KSCN and KSeCN gave furostanol derivatives **34** and **35**, respectively. Irradiation of **34** and **35** gave only a mixture that could not be isolated, resulting in failure to obtain a vitamin D derivative having a heterospiro ring in the side chain.

The cytotoxic activities of the synthetic vitamin D derivatives (**12**, **13**), spirostanol derivatives (**18**–**21**, **26**, **27**, **29** and **37**) and furostanol derivatives (**34**–**36**) against cancerous HCT 116 and Hep G2 cells were evaluated (Table 2). It is known that while HCT 116 cell lines scarcely express the MDR 1 (P-glycoprotein; P-gp) [22], Hep G2 cell lines overexpress it [23].

The cytotoxic effect of diosgenin **1** on Hep G2 cells was more potent than on HCT 116 cells. This is thought to be due to the difference in physiological properties between Hep G2 and HCT 116 cells. All synthesized vitamin D, spirostanol and furostanol derivatives mentioned above showed higher effects than **1** on both HCT 116 and Hep G2 cells. In particular, the furostanol derivatives **34** and **36** had markedly cytotoxic effects on HCT 116 cells, and spirostanol derivative **29** and furostanol derivative **36** had markedly potent cytotoxic effects on Hep G2 cells. Compound **36**, which had a furost-1α,3β-dihydroxy-26-thiocyano-6,8(14),20(22)-triene structure exhibited marked effects on both HCT 116 and Hep G2 cells. However, spirostanol **37**, which had the same structure of the A to D ring as **36** was less effective than **36**. In the case of the furostanol derivatives **34**–**36**, 6,8(14)-*trans*-diene **36** having a SCN group at the 26 position exhibited a greater cytotoxic effect than the 5,7-*cis*-dienes **34** and **35** having a SCN and SeCN group, respectively, at the same position, on both HCT 116 and Hep G2 cells. Thus, the presence of the 6,8(14)-*trans*-diene group and a SCN group at the 26 position in the furostanol structure may be important for producing a greater cytotoxic effect on both types of cells. Compound **36** had a markedly potent cytotoxic effect on not only HCT 116 cells weakly expressing P-pg but also P-gp overexpressing Hep G2 cells, which suggests that **36** is not influenced by efflux systems, such as P-gp in Hep G2 cells. This finding will be followed up by further investigations of more potent cytotoxic compounds that are not subject to the efflux systems of cancer cells.

The apoptosis-signaling pathway activated by the compounds in Hep G2 cell lines using the expression of *p53*, *Bax*, *p21* and *Bcl-2* mRNAs, was investigated. For this investigation, three different type of compounds, vitamin D type derivative **12**, spirostanol **26** and furostanol derivative **36** were selected.

Compounds **12** and **26** showed significant increases in the levels of apoptosis-related signals of *p53* and *Bax* mRNA expression, while the *p21* and *Bcl-2* mRNA expression remained unchanged (Table 3). This suggests that **12**- and **26**-induced apoptosis in Hep G2 cells is mediated via the activation of both *p53* and *Bax* mRNAs. These results are similar to the report by Chresta et al. [26]; the levels of the *p53* and *Bax* mRNAs were increased after etoposide-treatment of the testicular tumor line but there was no expression of the suppressor of apoptosis *Bcl-2* mRNA. In contrast, furostanol type derivative **36** overexpressed only *Bax* mRNA but not the other three mRNAs. *Bax* mRNA overexpression enhances cytochrome *c* release from mitochondria and induces apoptosis through a p53-independent pathway [27,28]. Takahashi et al. [29] reported that sufficient levels of *Bax* mRNA may bypass the need for upstream molecules such as *p53* mRNA in the process of chemotherapy-induced apoptosis. That report and our own results suggest that **36** is capable of mediating cell death in a p53-independent fashion. Perego et al. [30] reported that mutations of the *p53* gene play a role in the development of cisplatin resistance in cancer cells. This implies that **36**

may be a useful chemotherapeutical agent for the treatment of p53-deficient and/or *p53* mutant hepatoma.

5. Experimental

5.1. General procedures

Diosgenin, used as a starting material, was purchased from SIGMA Chemical Co. Ltd., USA. Commercial *p*-toluenesulfonyl chloride (TsCl) was recrystallized from chloroform/petroleum ether (1:5). Commercial Me_3N·HCl was dried for several minute prior to use. The other chemicals and solvents were of reagent grade and obtained from commercial suppliers. Melting points (m.p.) were determined using a Yanagimoto micromelting point apparatus and were uncorrected. Kieselgel 60 F_{254} (E. Merck) was used for the thin-layer chromatography (TLC). Spots were detected by spraying plates with 1:9 $Ce(SO_4)_2$–10% H_2SO_4 reagent, followed by heating the plate at 250 °C for 3 min. Preparative TLC was performed on 20 × 20 cm plates coated with a 1.0 mm layer of kieselgel 60 F_{254} (E. Merck). Column chromatography was carried out using a Kieselgel 60 column (70–230 mesh, E. Merck), and the eluates were monitored by TLC. An SSC-6300 HPLC instrument (Senshu Scientific Co. Ltd.) was employed for analytical HPLC (column, DOCOSIL, 10 × 250 mm; flow rate, 1.0 ml/min; column temperature, 40 °C, and DOCOSIL-B, 10 × 250 mm; flow rate, 1.0 ml/min; column temperature, 40 °C), attached to an SSC autoinjector 6310 and an SSC fraction collector 6320 for preparative HPLC. ^{1}H-NMR and ^{13}C-NMR at 500 and 125 MHz, respectively, as well as ^{1}H–^{1}H and ^{1}H–^{13}C COSY, DEPT, HMBC and NOE spectra were obtained with a JEOL JNM-A500 FT-NMR spectrometer. Tetramethylsilane was used as an internal standard. Chemical shifts are given in ppm. Multiplicities of the ^{1}H-NMR signals are indicated as s (singlet), d (doublet), dd (doublet of doublets), ddd (doublet of doublet of doublets), t (triplet), quin (quintet) and m (multiplet). Fast-atom-bombardment mass spectra (FABMS) and high resolution mass spectra (HRMS) were recorded on a JEOL JMS-DX 300 mass spectrometer.

5.1.1. Chemistry

*5.1.1.1. (20S,22R,25R)-Spirost-1α,3β-dihydroxy-5-ene (**20**) and (20S,22R,25R)-spirost-1α,3α-dihydroxy-5-ene (**21**).* A three-necked flask connected with a dropping funnel, a sealed mechanical stirrer and an inlet tube connected to an ammonia source was fitted in a cooling bath with liquid nitrogen in ethanol. After swepting with a stream of argon gas for 10 min, ammonia (160 ml) was trapped in the flask, then sodium pieces (10 g) was added under stirring. After further stirring for 20 min, the epoxide **19** (4.0 g, 9.4 mmol) in THF (90 ml) was added dropwise during 40 min. The cooling bath was removed and the mixture was stirred for 1 h. The flask was dipped into the cooling bath, and anhydrous ammonium chloride (NH_4Cl) (24 g) was added during 4 h. The cooling bath was removed and the mixture was stirred for 2 h at room temperature to turn white and pasty. After ammonia was removed by a stream of argon, ethanol was slowly dropped into the flask to decompose excessive sodium. The mixture was poured into 5% hydrochloric acid (200 ml), and extracted with CH_2Cl_2 (100 ml × 3). The organic extracts were washed with brine (200 ml), dried over Na_2SO_4 and filtered. The filtrate was evaporated to give a residue which was applied to a silica gel column (a gradient of 0–50% acetone in toluene) to give **20** (1.79 g, 44.1%) as white needles, m.p. 242–244 °C (after recrystallization from ethanol–water) (a white solid: m.p. > 230 °C [18]) and **21** (240 mg, 5.9%) as white needles, m.p. 245–248 °C (after recrystallization from ethanol–water). FABMS of **2**0: *m/z* 431 $[M + H]^+$; ^{1}H-NMR spectrum was agree with that of Liu et al. [18]; ^{13}C-NMR spectral data are listed in Table 1. HRMS (FAB) *m/z* calcd for $C_{27}H_{43}O_4$ $[M + H]^+$, 431.3161, found: 431.3153. FABMS of **21**: *m/z* 431 $[M + H]^+$; ^{1}H-NMR ($CDCl_3$) (only assignable signals are listed) δ 5.58 (1H, d, J = 5.2 Hz, H-6), 4.42 (1H, dd, J = 14.6, 7.3 Hz, H-16), 4.14 (1H, broad s, H-3β), 3.75 (1H, broad s, H-1β), 3.47 (1H, ddd, J = 11.0, 6.4, 1.8 Hz, H-26a), 3.37 (1H, t, J = 11.0 Hz, H-26b), 0.99 (3H, s, 19-CH_3), 0.97 (3H, d, J = 7.0 Hz, 21-CH_3), 0.79 (3H, s, 18-CH_3), 0.79 (3H, d, J = 6.1 Hz, 27-CH_3); ^{13}C-NMR spectral are listed in Table 1. HRMS (FAB): *m/z* calcd for $C_{27}H_{43}O_4$ $[M + H]^+$, 431.3161, found: 431.3151.

*5.1.1.2. (20S,22R,25R)-Spirost-1α,3β-diacetoxy-5-ene (**22**).* The general procedure for acetylation with acetic anhydride and pyridine was employed for **20** (4.91 g, 11.4 mmol) to afford **22** (5.46 g, 95.4%) as white needles, m.p. 171–173 °C (after recrystallization from ethanol–water). FABMS: *m/z* 515 $[M + H]^+$. ^{1}H-NMR ($CDCl_3$) (only assignable signals are listed) δ 5.53 (1H, d, J = 5.5 Hz, H-6), 5.05 (1H, broad s, H-1β), 4.91 (1H, m, H-3α), 4.41 (1H, dd, J = 15.0, 7.3 Hz, H-16), 3.47 (1H, ddd, J = 11.0, 4.3, 1.8 Hz, H-26a), 3.37 (1H, t, J = 11.0 Hz, H-26b), 2.05 (3H, s, $COCH_3$), 2.02 (3H, s, $COCH_3$), 1.10 (3H, s, 19-CH_3), 0.96 (3H, d, J = 6.7 Hz, 21-CH_3), 0.79 (3H, d, J = 6.4 Hz, 27-CH_3), 0.78 (3H, s, 18-CH_3); ^{13}C-NMR ($CDCl_3$) δ 170.3 ($\underline{C}OCH_3$), 170.3 ($\underline{C}OCH_3$), 136.1 (C-5), 124.9 (C-6), 109.3 (C-22), 80.7 (C-16), 74.5 (C-1), 69.3 (C-3), 66.8 (C-26), 62.1 (C-17), 56.4 (C-14), 42.1 (C-9), 41.6 (C-20), 40.5 (C-10), 40.3 (C-13), 39.5 (C-12), 37.3 (C-2), 31.9 (C-7), 31.8 (C-4), 31.8 (C-15), 31.4 (C-23), 31.3 (C-8), 30.3 (C-25), 28.8 (C-24), 21.3 ($CO\underline{C}H_3$), 21.1 ($CO\underline{C}H_3$), 20.1 (C-11), 19.4 (C-19), 17.1 (C-27), 16.3 (C-18), 14.5 (C-21). HRMS (FAB) *m/z* calcd for $C_{31}H_{47}O_6$ $[M + H]^+$, 515.3373, found: 515.3360.

*5.1.1.3. (20S,22R,25R)-Spirost-1α,3α-diacetoxy-5-ene (**23**).* The general procedure for acetylation with acetic anhydride and pyridine was employed for **21** (1.60 g, 3.72 mmol) to give **23** (1.50 g, 78.4%) as white needles, m.p. 191–193 °C (after recrystallization from acetone–water). FABMS: *m/z* 515 $[M + H]^+$; ^{1}H-NMR ($CDCl_3$) (only assignable signals are

listed) δ 5.47 (1H, d, J = 5.5 Hz, H-6), 5.00 (1H, broad s, H-3β), 4.89 (1H, broad s, H-1β), 4.42 (1H, dd, J = 15.0, 7.6 Hz, H-16), 3.47 (1H, ddd, J = 11.0, 4.6, 2.1 Hz, H-26a), 3.37 (1H, t, J = 11.0 Hz, H-26b), 2.03 (3H, s, $COCH_3$), 2.01 (3H, s, $COCH_3$), 1.08 (3H, s, 19-CH_3), 0.96 (3H, d, J = 7.0 Hz, 21-CH_3), 0.79 (3H, d, J = 7.9 Hz, 27-CH_3), 0.78 (3H, s, 18-CH_3); ^{13}C-NMR($CDCl_3$) δ 170.4 ($\underline{C}OCH_3$), 170.4 ($\underline{C}OCH_3$), 134.5 (C-5), 124.5 (C-6), 109.2 (C-22), 80.7 (C-16), 73.1 (C-1), 68.6 (C-3), 66.8 (C-26), 62.0 (C-17), 56.4 (C-14), 41.8 (C-9), 41.5 (C-20), 40.6 (C-10), 40.2 (C-13), 39.5 (C-12), 35.4 (C-4), 31.8 (C-7), 31.8 (C-15), 31.3 (C-23), 31.2 (C-8), 30.2 (C-25), 29.1 (C-2), 28.7 (C-24), 21.4 ($CO\underline{C}H_3$), 21.2 ($CO\underline{C}H_3$), 19.8 (C-11), 19.3 (C-19), 17.1 (C-27), 16.2 (C-18), 14.5 (C-21). HRMS (FAB): m/z calcd for $C_{31}H_{47}O_6$ $[M + H]^+$, 515.3373, found: 515.3363.

*5.1.1.4. (20S,22R,25R)-Spirost-1α,3β-diacetoxy-5,7-diene (**24**) and (20S,22R,25R)-spirost-1α,3β-diacetoxy-4,6-diene (**25**).* A mixture of diacetate **22** (1.10 g, 2.14 mmol) and *N*-bromosuccinimide (NBS) (495 mg, 2.78 mmol) in anhydrous *n*-hexane (50 ml) was refluxed for 2 h. After cooling to room temperature, the reaction mixture was filtered. The filtrate was evaporated to give a residue which was dissolved in xylene (50 ml). The mixture was combined with γ-collidine (8 ml) and refluxed for 2 h, and then filtered. The filtrate was diluted with diethyl ether (Et_2O) (200 ml). The mixture was washed with 5% aqueous HCl (100 ml) and brine (100 ml), dried over Na_2SO_4, and filtered. The filtrate was evaporated to give a residue which was subjected to silica gel column chromatography (a gradient of 0–10% ethyl acetate in *n*-hexane) to afford **24** (581 mg, 53.0%) as white needles, m.p. 195–196 °C (after recrystallization from acetone) and **25** (52 mg, 4.8%) as white needles, m.p. 152–154 °C (after recrystallization from acetone–H_2O). FABMS of **24**: m/z 513 $[M + H]^+$; ^{1}H-NMR ($CDCl_3$) (only assignable signals are listed) δ 5.67 (1H, dd, J = 6.1, 2.7 Hz, H-6), 5.39 (1H, quin, J = 2.7 Hz, H-7), 4.99 (1H, broad s, H-1β), 4.99 (1H, m, H-3α), 4.51 (1H, dd, J = 14.3, 7.6 Hz, H-16), 3.48 (1H, ddd, J = 11.0, 4.3, 1.8 Hz, H-26a), 3.38 (1H, t, J = 11.0 Hz, H-26b), 2.43 (1H, m, H-9), 2.09 (3H, s, $COCH_3$), 2.04 (3H, s, $COCH_3$), 1.03 (3H, s, 19-CH_3), 0.98 (3H, d, J = 6.7 Hz, 21-CH_3), 0.80 (3H, d, J = 6.4 Hz, 27-CH_3), 0.72 (3H, s, 18-CH_3); ^{13}C-NMR($CDCl_3$) δ 170.4 ($\underline{C}OCH_3$), 170.3 ($\underline{C}OCH_3$), 139.9 (C-8), 135.2 (C-5), 121.5 (C-6), 115.6 (C-7), 109.3 (C-22), 80.5 (C-16), 74.2 (C-1), 68.4 (C-3), 66.9 (C-26), 61.9 (C-17), 54.4 (C-14), 42.1 (C-20), 41.1 (C-10), 40.9 (C-13), 38.9 (C-12), 37.7 (C-9), 35.8 (C-2), 32.0 (C-4), 31.4 (C-23), 30.9 (C-15), 30.3 (C-25), 28.8 (C-24), 21.3 ($CO\underline{C}H_3$), 21.2 ($CO\underline{C}H_3$), 20.1 (C-11), 17.1 (C-27), 16.4 (C-18), 16.2 (C-19), 14.5 (C-21); HRMS (FAB) m/z calcd for $C_{31}H_{45}O_6$ $[M + H]^+$, 513.3216, found: 513.3205. FABMS of **25**: m/z 513 $[M + H]^+$; ^{1}H-NMR ($CDCl_3$) (only assignable signals are listed) δ 5.98 (1H, dd, J = 9.8, 2.8 Hz, H-6), 5.68 (1H, dd, J = 9.8, 1.5 Hz, H-7), 5.43 (1H, broad s, H-3α), 5.42 (1H, broad s, H-4), 5.03 (1H, broad s, H-1β), 4.43 (1H, dd, J = 14.7, 7.6 Hz, H-16), 3.47 (1H, ddd, J = 11.0, 4.0, 1.5 Hz, H-26a), 3.37 (1H, t, J = 11.0 Hz, H-26b), 2.06 (3H, s, $COCH_3$), 2.03 (3H, s, $COCH_3$), 1.09 (3H, s, 19-CH_3), 0.96 (3H, d, J = 7.0 Hz, 21-CH_3), 0.84 (3H, s, 18-CH_3), 0.79 (3H, d, J = 6.1 Hz, 27-CH_3); ^{13}C-NMR($CDCl_3$) δ 170.7 ($\underline{C}OCH_3$), 170.6 ($\underline{C}OCH_3$), 142.5 (C-5), 131.5 (C-7), 127.9 (C-6), 121.1 (C-4), 109.2 (C-22), 80.5 (C-16), 73.9 (C-1), 68.0 (C-3), 66.8 (C-26), 62.0 (C-17), 53.8 (C-14), 44.5 (C-9), 41.5 (C-13), 41.4 (C-20), 39.6 (C-12), 38.2 (C-10), 36.7 (C-8), 31.3 (C-15), 31.3 (C-23), 30.2 (C-25), 28.7 (C-24), 28.4 (C-2), 21.2 ($CO\underline{C}H_3$), 21.0 ($CO\underline{C}H_3$), 20.1 (C-11), 19.0 (C-19), 17.1 (C-27), 16.3 (C-18), 14.4 (C-21). HRMS (FAB): m/z calcd for $C_{31}H_{45}O_6$ $[M + H]^+$, 513.3216, found: 513.3215.

*5.1.1.5. (20S,22R,25R)-Spirost-1α,3β-dihydroxy-5,7-diene (**26**).* A solution of compound **24** (270 mg, 0.53 mmol) in MeOH (8 ml) and benzene (9 ml) was combined with methanolic 2 M KOH (10 ml). The mixture was stirred at 65 °C for 1 h, and then poured into ice-cold water (100 ml) and extracted with CH_2Cl_2 (70 ml × 3). The organic extracts were washed with brine (150 ml), dried and evaporated to give a residue which was recrystallized from ethanol–water to afford **26** (210 mg, 92.9%) as white needles (m.p. 233–234 °C, recrystallizaion from acetone). FABMS: m/z 429 $[M + H]^+$; ^{1}H-NMR ($CDCl_3$) (only assignable signals are listed) δ 5.70 (1H, dd, J = 5.8, 2.4 Hz, H-6), 5.37 (1H, quin, J = 2.4 Hz, H-7), 4.50 (1H, dd, J = 14.5, 7.6 Hz, H-16), 4.05 (1H, m, H-3α), 3.75 (1H, broad s, H-1β), 3.48 (1H, ddd, J = 11.0, 4.3, 2.1 Hz, H-26a), 3.39 (1H, t, J = 11.0 Hz, H-26b), 2.73 (1H, m, H-9), 0.99 (3H, d, J = 6.7 Hz, 21-CH_3), 0.95 (3H, s, 19-CH_3), 0.80 (3H, d, J = 6.4 Hz, 27-CH_3), 0.74 (3H, s, 18-CH_3); ^{13}C-NMR spectral data are listed in Table 1. HRMS (FAB): m/z calcd for $C_{27}H_{41}O_4$ $[M + H]^+$, 429.3005, found: 429.2977.

*5.1.1.6. (20S,22R,25R)-Spirost-1α,3β-dihydroxy-4,6-diene (**27**).* The same reaction of 4,6-dien diacetate **25** (64 mg, 0.12 mmol) as described for **26** gave **27** (42 mg, 75.8%) as white needles (m.p. 208–210 °C, recrystallization from acetone). FABMS: m/z 429 $[M + H]^+$; ^{1}H-NMR ($CDCl_3$) (only assignable signals are listed) δ 5.97 (1H, dd, J = 9.8, 2.4 Hz, H-6), 5.64 (1H, d, J = 9.8 Hz, H-7), 5.48 (1H, broad s, H-4), 4.52 (1H, m, H-3α), 4.43 (1H, dd, J = 15.0, 7.6 Hz, H-16), 3.89 (1H, broad s, H-1β), 3.47 (1H, ddd, J = 11.0, 4.3, 1.5 Hz, H-26a), 3.37 (1H, t, J = 11.0 Hz, H-26b), 1.02 (3H, s, 19-CH_3), 0.97 (3H, d, J = 7.0 Hz, 21-CH_3), 0.85 (3H, s, 18-CH_3), 0.79 (3H, d, J = 6.4 Hz, 27-CH_3); ^{13}C-NMR spectral date are listed in Table 1. HRMS (FAB): m/z calcd for $C_{27}H_{41}O_4$ $[M + H]^+$, 429.3005, found: 429.2998.

*5.1.1.7. (20S,22R,25R)-Spirost-1α,3α-diacetoxy-5,7-diene (**28**).* The same reaction and procedure for **23** (100 mg, 0.194 mmol) as described for **24** gave **28** (21 mg, 25.1%) as a white needles, m.p. 152–155 °C (after recrystallization from acetone–H_2O). FABMS: m/z 513 $[M + H]^+$; ^{1}H-NMR ($CDCl_3$) (only assignable signals are listed) δ 5.68 (1H, dd, J = 5.5, 2.4 Hz, H-6), 5.43 (1H, quin, J = 2.4 Hz, H-7), 5.08 (1H, broad s, H-3β), 4.89 (1H, m, H-1β), 4.51 (1H, dd,

J = 14.3, 7.6 Hz, H-16), 3.49 (1H, ddd, *J* = 11.0, 4.6, 1.8 Hz, H-26a), 3.39 (1H, t, *J* = 11.0 Hz, H-26b), 2.57 (1H, m, H-9), 2.07 (3H, s, $COCH_3$), 2.02 (3H, s, $COCH_3$), 1.02 (3H, s, 19-CH_3), 0.99 (3H, d, *J* = 6.7 Hz, 21-CH_3), 0.80 (3H, d, *J* = 6.4 Hz, 27-CH_3), 0.72 (3H, s, 18-CH_3); ^{13}C-NMR($CDCl_3$) δ 170.5 ($\underline{C}OCH_3$), 170.4 ($\underline{C}OCH_3$), 139.9 (C-8), 134.2 (C-5), 121.2 (C-6), 115.8 (C-7), 109.3 (C-22), 80.5 (C-16), 72.5 (C-1), 67.7 (C-3), 66.8 (C-26), 61.9 (C-17), 54.5 (C-14), 42.0 (C-20), 41.0 (C-10), 40.9 (C-13), 38.8 (C-12), 37.7 (C-9), 34.1 (C-4), 31.3 (C-23), 30.8 (C-15), 30.2 (C-25), 30.0 (C-2), 28.8 (C-24), 21.4 ($CO\underline{C}H_3$), 21.3 ($CO\underline{C}H_3$), 20.0 (C-11), 17.1 (C-27), 16.4 (C-19), 16.3 (C-18), 14.5 (C-21). HRMS (FAB): *m/z* calcd for $C_{31}H_{45}O_6$ $[M + H]^+$, 513.3216, found: 513.3199.

*5.1.1.8. (20S,22R,25R)-Spirost-1α,3α-dihydroxy-5,7-diene (**29**).* The reaction of **28** (210 mg, 0.41 mmol) as described for **26** gave **29** (142 mg, 80.9%) as white needles, m.p. 212–214 °C (recrystallization from acetone). FABMS: *m/z* 429 $[M + H]^+$; ^{1}H-NMR ($CDCl_3$) (only assignable signals are listed) δ 5.71 (1H, dd, *J* = 5.5, 2.4 Hz, H-6), 5.37 (1H, quin, *J* = 2.4 Hz, H-7), 4.51 (1H, dd, *J* = 14.6, 7.3 Hz, H-16), 4.23 (1H, broad s, H-3β), 3.63 (1H, broad s, H-1β), 3.49 (1H, ddd, *J* = 11.0, 4.6, 1.8 Hz, H-26a), 3.40 (1H, t, *J* = 11.0 Hz, H-26b), 3.06 (1H, m, H-9), 0.98 (3H, d, *J* = 6.7 Hz, 21-CH_3), 0.92 (3H, s, 19-CH_3), 0.80 (3H, d, *J* = 7.1 Hz, 27-CH_3), 0.72 (3H, s, 18-CH_3); ^{13}C-NMR spectral data are listed in Table 1. HRMS (FAB): *m/z* calcd for $C_{27}H_{41}O_4$ $[M + H]^+$, 429.3005, found: 429.2997.

*5.1.1.9. (20S,22R,25R)-9,10-Secospirosta-5,7,10(19)-triene-1α,3β-diol (**12**).* Irradiation of **26** (120 mg, 0.28 mmol) in Et_2O (600 ml) with a 200 W high pressure mercury lamp (UV-LT10) (Ishii Sho-ten Co., Ltd., Tokyo, Japan) through a Pyrex filter (No. 7740) was performed according to the method reported by Morisaki et al. [10] to give **12** (24 mg, 20.0%) as a colorless foam. FABMS: *m/z* 429 $[M + H]^+$; ^{1}H-NMR ($CDCl_3$) (only assignable signals are listed) δ 6.34 (1H, d, *J* = 11.3 Hz, H-6), 6.00 (1H, d, *J* = 11.3 Hz, H-7), 5.29 (1H, d, *J* = 1.8 Hz, H-19a), 4.97 (1H, d, *J* = 1.8 Hz, H-19b), 4.48 (1H, dd, *J* = 15.0, 7.3 Hz, H-16), 4.42 (1H, dd, *J* = 7.3, 4.3 Hz, H-1β), 4.23 (1H, m, H-3α), 3.48 (1H, ddd, *J* = 11.0, 4.3, 2.1 Hz, H-26a), 3.38 (1H, t, *J* = 11.0 Hz, H-26b), 0.98 (3H, d, *J* = 7.0 Hz, 21-CH_3), 0.79 (3H, d, *J* = 6.4 Hz, 27-CH_3), 0.67 (3H, s, 18-CH_3); ^{13}C-NMR spectral data are listed in Table 1. HRMS (FAB): *m/z* calcd for $C_{27}H_{41}O_4$ $[M + H]^+$, 429.3005, found: 429.2990.

*5.1.1.10. (20S,22R,25R)-9,10-Secospirosta-5,7,10(19)-triene-1α,3α-diol (**13**).* The same reaction of **29** (50 mg, 0.12 mmol) as described for **12** gave **13** (10 mg, 20.0%) as a colorless foam. FABMS: *m/z* 429 $[M + H]^+$; ^{1}H-NMR ($CDCl_3$) (only assignable signals are listed) δ 6.41 (1H, d, *J* = 11.3 Hz, H-6), 6.02 (1H, d, *J* = 11.3 Hz, H-7), 5.25 (1H, d, *J* = 1.8 Hz, H-19a), 4.97 (1H, d, *J* = 1.8 Hz, H-19b), 4.49 (1H, dd, *J* = 15.0, 7.3 Hz, H-16), 4.32 (1H, broad s, H-1β), 4.05 (1H, broad s, H-3β), 3.48 (1H, ddd, *J* = 11.0, 4.3, 2.1 Hz, H-26a), 3.38 (1H, t, *J* = 11.0 Hz, H-26b), 0.98 (3H, d, *J* = 7.0 Hz, 21-CH_3), 0.79 (3H, d, *J* = 6.4 Hz, 27-CH_3), 0.66 (3H, s, 18-CH_3); ^{13}C-NMR spectral data are listed in Table 1. HRMS (FAB): *m/z* calcd for $C_{27}H_{41}O_4$ $[M + H]^+$, 429.3005, found: 429.2987.

*5.1.1.11. (25R)-Furost-1α,3β,26-triacetoxy-5,7,20(22)-triene (**30**).* A solution of **24** (927 mg, 1.81 mmol) in acetic anhydride (30 ml) was heated in a bomb tube at 180 °C for 8 h. The reaction mixture was coevaporated with toluene to afford a residue which was subjected to column chromatography (a gradient of 0–10% ethyl acetate in *n*-hexane) to give compound **30** (785 mg, 78.3%) as a colorless foam. FABMS: *m/z* 555 $[M + H]^+$; ^{1}H-NMR ($CDCl_3$) (only assignable signals are listed) δ 5.68 (1H, dd, *J* = 5.5, 2.1 Hz, H-6), 5.43 (1H, quin, *J* = 2.1 Hz, H-7), 5.00 (1H, brs, H-1β), 5.00 (1H, m, H-3α), 4.83 (1H, ddd, *J* = 10.1, 7.9, 5.8 Hz, H-16), 3.94 (1H, dd, *J* = 10.7, 5.8 Hz, H-26a), 3.88 (1H, dd, *J* = 10.7, 6.4 Hz, H-26b), 2.61 (1H, d, *J* = 10.1 Hz, H-17), 2.44 (1H, m, H-9), 2.09 (3H, $COCH_3$), 2.05 (3H, $COCH_3$), 2.04 (3H, $COCH_3$), 1.59 (3H, s, 21-CH_3), 1.03 (3H, s, 19-CH_3), 0.94 (3H, d, *J* = 6.7 Hz, 27-CH_3), 0.63 (3H, s, 18-CH_3); ^{13}C-NMR($CDCl_3$) δ 171.3 ($\underline{C}OCH_3$), 170.4 ($\underline{C}OCH_3$), 170.3 ($\underline{C}OCH_3$), 151.8 (C-22), 139.5 (C-8), 135.4 (C-5), 121.5 (C-6), 115.9 (C-7), 103.9 (C-20), 84.0 (C-16), 74.3 (C-1), 69.2 (C-26), 68.4 (C-3), 64.0 (C-17), 52.8 (C-14), 43.7 (C-13), 41.1 (C-10), 38.8 (C-12), 37.8 (C-9), 35.8 (C-2), 33.2 (C-23), 32.1 (C-25), 32.0 (C-4), 30.8 (C-15), 23.2 (C-24), 21.3 ($CO\underline{C}H_3$), 21.2 ($CO\underline{C}H_3$), 21.0 ($CO\underline{C}H_3$), 20.3 (C-11), 16.7 (C-27), 16.2 (C-19), 14.0 (C-18), 11.5 (C-21). HRMS (FAB) *m/z* calcd for $C_{33}H_{47}O_7$ $[M + H]^+$: 555.3322, found: 555.3307.

*5.1.1.12. (25R)-Furost-1α,3β-dihydroxy-26-p-toluenesulfonyloxy-5,7,20(22)-triene (**32**).* A solution of triene acetate **30** (280 mg, 0.51 mmol) in methanolic 2 M KOH solution (8 ml) was stirred at room temperature for 1 h. Then the reaction mixture was poured into 5% $NaHCO_3$ aqueous solution (80 ml) and extracted with CH_2Cl_2 (50 ml × 3). The extracts were washed with brine (150 ml), dried over Na_2SO_4 and filtered. The filtrate was evaporated to give a crude triol **31** (213 mg). FABMS: *m/z* 429 $[M + H]^+$; HRMS (FAB) *m/z* calcd for $C_{27}H_{41}O_4$ $[M + H]^+$, 429.3005, found: 429.2993. A solution of **31** (213 mg) in anhydride CH_2Cl_2 (4.6 ml) was added to a stirred solution of Et_3N (0.14 ml, 1.02 mmol), Me_3N.HCl (5 mg, 0.05 mmol) and *p*-toluenesulfonylchloride (TsCl) (116 mg, 0.61 mmol) in anhydrous CH_2Cl_2 (1 ml) at 0 °C, and the mixture was stirred for 10 min. Ice-cold water (80 ml) was added to the mixture, followed by extraction with CH_2Cl_2 (50 ml × 3). The organic extracts were washed successively with 5% HCl (150 ml), 5% $NaHCO_3$ (100 ml) and brine (200 ml), dried over Na_2SO_4 and filtered. The filtrate was evaporated to give a residue which was purified by silica gel chromatography (a gradient of 0–25% acetone in toluene) to obtain the tosylate **32** (204 mg, 69.4%) as a yellowish foam. FABMS: *m/z* 583 $[M + H]^+$; ^{1}H-NMR ($CDCl_3$) (only assignable signals are listed) δ 7.78 (2H, d, *J* = 7.9 Hz, C_6H_4),

7.34 (2H, d, J = 7.9 Hz, C_6H_4), 5.70 (1H, dd, J = 5.5, 1.8 Hz, H-6), 5.40 (1H, quin, J = 1.8 Hz, H-7), 4.78 (1H, ddd, J = 10.1, 7.6, 5.8 Hz, H-16), 4.06 (1H, m, H-3α), 3.89 (1H, dd, J = 9.5, 5.2 Hz, H-26a), 3.80 (1H, dd, J = 9.5, 6.7 Hz, H-26b), 3.76 (1H, broad s, H-1β), 2.75 (1H, m, H-9), 2.58 (1H, d, J = 10.1 Hz, H-17), 2.44 (3H, s, C_6H_4-C$\underline{H}_3$), 1.54 (3H, s, 21-CH_3), 0.95 (3H, s, 19-CH_3), 0.90 (3H, d, J = 6.7 Hz, 27-CH_3), 0.60 (3H, s, 18-CH_3); ^{13}C-NMR($CDCl_3$) δ 151.2 (C-22), 144.6 (a phenyl carbon), 139.5 (C-8), 136.5 (C-5), 133.0, 129.8 and 127.8 (phenyl carbons), 121.7 (C-6), 115.6 (C-7), 104.2 (C-20), 84.0 (C-16), 74.8 (C-26), 72.6 (C-1), 65.2 (C-3), 63.9 (C-17), 52.8 (C-14), 43.8 (C-13), 42.3 (C-10), 39.9 (C-4), 38.9 (C-12), 38.4 (C-2), 37.6 (C-9), 33.1 (C-23), 32.3 (C-25), 30.0 (C-15), 22.9 (C-24), 21.6 (C_6H_4–$\underline{C}H_3$), 20.8 (C-11), 16.4 (C-27), 16.3 (C-19), 13.9 (C-18), 11.4 (C-21). HRMS (FAB) m/z calcd for $C_{34}H_{47}O_6S$ $[M + H]^+$, 583.3093, found: 583.3110.

*5.1.1.13. (25R)-Furost-1α,3β-dihydroxy-26-thiocyano-5,7,20(22)-triene (**34**).* Sodium iodide (227 mg, 1.51 mmol) was added to a solution of tosylate **32** (440 mg, 0.76 mmol) in 3-pentanone (14 ml), and the mixture was stirred at 70 °C for 7 h. The reaction mixture was poured into ice-cold water (100 ml), and extracted with CH_2Cl_2 (80 ml × 3). The organic extracts were washed with 10% $Na_2S_2O_3$ aqueous solution (100 ml × 2) and brine (200 ml), and dried over anhydrous Na_2SO_4. After filtration, the filtrate was evaporated under vacuum to give crude compound **33** (419 mg). FABMS: m/z 538 $[M]^+$; ^{1}H-NMR ($CDCl_3$) (only assignable signals are listed) δ 5.73 (1H, dd, J = 5.8, 2.8 Hz, H-6), 5.42 (1H, quin, J = 2.8 Hz, H-7), 4.84 (1H, ddd, J = 10.1, 7.9, 5.8 Hz, H-16), 4.07 (1H, m, H-3α), 3.78 (1H, broad s, H-1β), 3.25 (1H, dd, J = 9.8, 4.3 Hz, H-26a), 3.16 (1H, dd, J = 9.8, 5.8 Hz, H-26b), 2.62 (1H, d, J = 10.1 Hz, H-17), 1.61 (3H, s, 21-CH_3), 0.99 (3H, d, J = 6.4 Hz, 27-CH_3), 0.97 (3H, s, 19-CH_3), 0.65 (3H, s, 18-CH_3); HRMS (FAB) m/z calcd for $C_{27}H_{39}O_3I$ $[M]^+$, 538.1944, found: 538.1935. To a solution of crude **33** (419 mg, 0.78 mmol) in DMF (22 ml) was added KSCN (152 mg, 1.56 mmol), and the mixture was stirred at 70 °C for 1.5 h. The reaction mixture was poured into ice-water (100 ml), and extracted with CH_2Cl_2 (80 ml × 3). The organic extracts were washed with brine (200 ml) and dried over Na_2SO_4. After filtration, the filtrate was evaporated under vacuum to give a residue which was purified by preparative HPLC (35% H_2O–acetone) to afford compound **34** (135 mg, 38.1% from **32**) as a colorless foam. FABMS: m/z 469 $[M]^+$; ^{1}H-NMR ($CDCl_3$) (only assignable signals are listed) δ 5.71 (1H, dd, J = 5.5, 2.1 Hz, H-6), 5.41 (1H, quin, J = 2.1 Hz, H-7), 4.84 (1H, ddd, J = 10.1, 7.6, 5.8 Hz, H-16), 4.06 (1H, m, H-3α), 3.76 (1H, broad s, H-1β), 3.03 (1H, dd, J = 12.8, 5.5 Hz, H-26a), 2.80 (1H, dd, J = 12.8, 7.6 Hz, H-26b), 2.75 (1H, m, H-9), 2.63 (1H, d, J = 10.1 Hz, H-17), 1.61 (3H, s, 21-CH_3), 1.07 (3H, d, J = 6.7 Hz, 27-CH_3), 0.95 (3H, s, 19-CH_3), 0.64 (3H, s, 18-CH_3); ^{13}C-NMR spectral data are listed in Table 1. HRMS (FAB) m/z calcd for $C_{28}H_{39}O_3NS$ $[M]^+$, 469.2651, found: 469.2658.

*5.1.1.14. (25R)-Furost-1α,3β-dihydroxy-26-selenocyano-5,7,20(22)-triene (**35**).* The similar reaction of crude **33** (174 mg, 0.78 mmol) derived from **32** with KSeCN (93 mg, 0.64 mmol) described above gave compound **35** (40 mg, 24%) as a colorless foam. FABMS: m/z 517 $[M]^+$; ^{1}H-NMR ($CDCl_3$) (only assignable signals are listed) δ 5.71 (1H, dd, J = 5.5, 2.1 Hz, H-6), 5.41 (1H, quin, J = 2.1 Hz, H-7), 4.84 (1H, ddd, J = 10.1, 7.6, 5.5 Hz, H-16), 4.05 (1H, m, H-3α), 3.76 (1H, broad s, H-1β), 3.16 (1H, dd, J = 11.9, 5.5 Hz, H-26a), 2.96 (1H, dd, J = 11.9, 7.3 Hz, H-26b), 2.75 (1H, m, H-9), 2.63 (1H, d, J = 10.1 Hz, H-17), 1.61 (3H, s, 21-CH_3), 1.08 (3H, d, J = 6.7 Hz, 27-CH_3), 0.95 (3H, s, 19-CH_3), 0.64 (3H, s, 18-CH_3); ^{13}C-NMR spectral data are listed in Table 1. HRMS (FAB) m/z calcd for $C_{28}H_{39}O_3NSe$ $[M]^+$, 517.2095, found: 517.2098.

*5.1.1.15. (25R)-Furost-1α,3β-dihydroxy-26-thiocyano-6,8(14),20(22)-triene (**36**) and (20S,22R,25R)-spirost-1α,3β-dihydroxy-6,8(14)-diene (**37**).* A solution of crude **31** (378 mg, 0.83 mmol) derived from **30** in pyridine (8 ml) was added with TsCl (473 mg, 2.49 mmol) in pyridine (3 ml), then stirred at 0 °C for 1.5 h. The mixture was poured into ice-cold water (100 ml) and extracted with CH_2Cl_2 (80 ml × 3). The organic extracts were washed with 5% HCl (100 ml × 3), 5% $NaHCO_3$ solution (200 ml) and brine (250 ml), dried over Na_2SO_4 and filtered. The filtrate was evaporated to give a residue (363 mg) which was dissolved in 3-pentanone (12 ml) and mixed with NaI (187 mg, 1.25 mmol). The mixture was stirred at 70 °C for 5 h. The reaction mixture was then poured into ice-cold water (50 ml) and extracted with CH_2Cl_2 (30 ml × 3). The organic extracts were washed with 10% $Na_2S_2O_3$ aqueous solution (80 ml × 2) and brine (100 ml), and dried over anhydrous Na_2SO_4. After filtration, the filtrate was evaporated to give a crude iodide (342 mg). A solution of the crude iodide (342 mg, 0.64 mmol) in DMF (20 ml) was mixed with KSCN (124 mg, 1.28 mmol), and the mixture was stirred at 70 °C for 2 h. The reaction mixture was poured into ice-water (50 ml), and extracted with CH_2Cl_2 (50 ml × 3). The organic extracts were washed with brine (150 ml) and dried over Na_2SO_4. After filtration, the filtrate was evaporated to give a residue which was subjected to preparative HPLC (30% H_2O–acetone) to give compounds **36** (colorless foam, 38 mg, 9.7%, from **30**) and **37** (white needles, m.p. 201–203 °C, after recrystallization from acetone, 45 mg, 12.6%, from **30**). FABMS of **36**: m/z 469 $[M]^+$; ^{1}H-NMR ($CDCl_3$) (only assignable signals are listed) δ 6.12 (1H, dd, J = 9.8, 3.1 Hz, H-7), 5.35 (1H, dd, J = 9.8, 1.6 Hz, H-6), 4.82 (1H, ddd, J = 10.1, 8.2, 4.6 Hz, H-16), 4.08 (1H, m, H-3α), 3.90 (1H, broad s, H-1β), 3.04 (1H, dd, J = 12.8, 5.5 Hz, H-26a), 2.81 (1H, dd, J = 12.8, 7.6 Hz, H-26b), 2.67 (1H, d, J = 14.0 Hz, H-5), 2.59 (1H, d, J = 10.1 Hz, H-17), 1.63 (3H, s, 21-CH_3), 1.08 (3H, d, J = 6.7 Hz, 27-CH_3), 0.95 (3H, s, 18-CH_3), 0.67 (3H, s, 19-CH_3); ^{13}C-NMR spectral data are listed in Table 1. HRMS (FAB) of **36** m/z calcd for $C_{28}H_{39}O_3NS$ $[M]^+$, 469.2651, found: 469.2645. FABMS of **37**: m/z 429 $[M + H]^+$; ^{1}H-NMR ($CDCl_3$) (only assignable signals were listed) δ 6.12 (1H, dd,

J = 9.8, 3.1 Hz, H-7), 5.33 (1H, dd, J = 9.8, 1.5 Hz, H-6), 4.38 (1H, dd, J = 15.0, 7.6 Hz, H-16), 4.08 (1H, m, H-3α), 3.89 (1H, broad s, H-1β), 3.48 (1H, ddd, J = 10.7, 4.0, 1.8 Hz, H-26a), 3.39 (1H, t, J = 10.7 Hz, H-26b), 1.04 (3H, s, 18-CH_3), 0.99 (3H, d, J = 6.4 Hz, 21-CH_3), 0.80 (3H, d, J = 6.3 Hz, 27-CH_3), 0.67 (3H, s, 19-CH_3); ^{13}C-NMR spectral data are listed in Table 1. HRMS (FAB) of **37** *m/z* calcd for $C_{27}H_{41}O_4$ $[M + H]^+$, 429.3005, found: 429.2980.

*5.1.1.16. (25R)-Furost-1α,3β-dihydroxy-26-p-toluenesulfonyloxy -6,8(14),20(22)-triene (**38**).* To a solution of the 5,7-diene tosylate **32** (45 mg, 0.08 mmol) in chloroform (2 ml) was added 3 M HCl–methanol (8 ml) followed by stirring at room temperature for 20 h. Then the reaction mixture was poured into 5% $NaHCO_3$ aqueous solution (30 ml), and extracted with CH_2Cl_2 (30 ml × 3). The extracts were washed with brine (100 ml), dried over Na_2SO_4 and evaporated to give a residue which was purified by silica gel column chromatography (a gradient of 0–15% acetone in toluene) to afford **38** (22 mg, 48.9%) as a colorless foam. FABMS: *m/z* 583 $[M + H]^+$; ^{1}H-NMR ($CDCl_3$) (only assignable signals are listed) δ 7.78 (2H, d, J = 7.9 Hz, C_6H_4), 7.34 (2H, d, J = 7.9 Hz, C_6H_4), 6.12 (1H, dd, J = 9.8, 3.4 Hz, H-7), 5.33 (1H, dd, J = 9.8, 1.5 Hz, H-6), 4.75 (1H, ddd, J = 10.1, 7.6, 5.8 Hz, H-16), 4.09 (1H, m, H-3β), 3.90 (1H, dd, J = 9.5, 4.9 Hz, H-26a), 3.89 (1H, broad s, H-1α), 3.81 (1H, dd, J = 9.5, 6.4 Hz, H-26b), 2.64 (1H, d, J = 15.0 Hz, H-5), 2.59 (1H, d, J = 10.1 Hz, H -17), 2.44 (3H, s, C_6H_4-$C\underline{H}_3$), 1.56 (3H, s, 21-CH_3), 0.91 (3H, s, 18-CH_3), 0.91 (3H, d, J = 6.7 Hz, 27-CH_3), 0.67 (3H, s, 19-CH_3); ^{13}C-NMR($CDCl_3$) δ 151.3 (C-22), 144.6 (C-14), 144.6 and 133.1 (phenyl carbons), 130.5 (C-6), 129.8 and 127.8 (phenyl carbons), 126.2 (C-8), 124.6 (C-7), 104.2 (C-20), 83.7 (C-16), 74.8 (C-26), 72.4 (C-1), 66.8 (C-3), 64.0 (C-17), 43.5 (C-13), 41.9 (C-9), 39.8 (C-10), 38.4 (C-2), 36.9 (C-5), 36.4 (C-4), 36.4 (C-12), 34.5 (C-15), 32.9 (C-23), 32.3 (C-25), 22.9 (C-24), 22.2 (C-18), 21.6 (C_6H_4–$\underline{C}H_3$), 19.0 (C-11), 16.3 (C-27), 12.2 (C-19), 11.7 (C-21). HRMS (FAB) *m/z* calcd for $C_{34}H_{47}O_6S$ $[M + H]^+$, 583.3093, found: 583.3079.

*5.1.1.17. (25R)-Furost-1α,3β-dihydroxy-26-thiocyano-6,8(14),20(22)-triene (**36**) from **38**.* The general procedure for iodination was employed for **38** (18 mg, 0.03 mmol) to give crude **39** (15 mg), then the same reaction of **39** with KSCN (6 mg, 0.06 mmol) as described for **35** gave compound **36** (11 mg, 75.8%). The FABMS spectrum and ^{1}H- and ^{13}C-NMR spectra of **36** were agreed with those of the compound obtained from **31**.

5.1.2. Cytotoxic activities

5.1.2.1. Cell lines and culture. The human colorectal carcinoma cell line (HCT 116, ATCC No. CCL-247) and human hepatoma cell line (Hep G2 No. RCB0459) were purchased from Dainippon Pharmaceutical Co. Ltd. (Osaka, Japan) and RIKEN Cell Bank (Tsukuba, Japan), respectively. Dulbecco's modified Eagle's medium (DMEM), McCoy's 5A medium, fetal bovine serum (FBS) and penicillin–streptomycin mixture (100 U/ml penicillin and 100 μg/ml streptomycin) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan), Sigma (MO, USA), Biosource International (CA, USA) and Bio Whittaker (ND, USA), respectively. The HCT 116 cells were maintained in McCoy's 5A medium and Hep G2 cells were cultured in DMEM. Each medium was supplemented with 10% FBS and a penicillin–streptomycin mixture at 37 °C in a humidified atmosphere containing 5% CO_2 in air.

5.1.2.2. Cytotoxicity test. Aliquots (200 μl) of 5×10^3 cells per ml of HCT 116 and Hep G2 cells were seeded in 96 well flat-bottomed plates (Microtest^{TM} Tissue Culture Plate, 96 Well, Flat Bottom with Low Evaporation Ltd., Falcon, NJ, USA), and were incubated in a medium containing 10% FBS and a penicillin–streptomycin mixture at 37 °C in a humidified atmosphere of 5% CO_2 for 24 h. The test drugs were dissolved in dimethyl sulfoxide (DMSO). The incubation medium was replaced with each test medium giving a final concentration of 1–500 μmol/l of test compounds and no drug in 2 μl DMSO over 2 days.

The ability of the drug to inhibit cellular growth was determined using the MTT assay [25]. The cytotoxic activities of the test drugs were determined as previously described [31]. Each experiment was performed in duplicate wells, and all experiments involving control (DMSO only) and the drug treatments were performed separately three to five times. Data represent mean ± S.D. values.

5.1.3. Semi-quantitative RT-PCR analysis for apoptosis-related signals

5.1.3.1. mRNAs. Hep G2 cells (1×10^6) were seeded in 10 cm dishes. Each dish was cultured with 12 ml of DMEM containing 10% FBS and a penicillin–streptomycin mixture at 37 °C in a humidified atmosphere containing 5% CO_2 in air.

After 24 h, the medium was replaced with test medium containing vitamin D-type derivative **12**, spirostanol **26** and furostanol **36**. Hep G2 cells were treated with these test drugs at their IC_{50} values for 48 h. All experiments were performed separately at least three times. Samples of mRNA were obtained from each culture at 48 h using a total RNA extraction kit (Rneasy Mini Kit) (Qiagen GmbH, Hilden, Germany). The RT-PCR primers were 5′-GCA CTG GTG TTT TGT TGT GG-3′ and 5′-GTG GTT TCA AGG CCA GAT GT-3′ for *p53* primers (304 bp), 5′-AAG CTG AGC GAG TGT CTC AAG CGC-3′ and 5′-ACC ACT GTG ACC TGC TCC AGA AG-3′ for *Bax* primers (424 bp), 5′-GAC ACC ACT GGA GGG TGA CT-3′ and 5′-CAG GTC CAC ATG GTC TTC CT-3′ for *p21* primers (172 bp), 5′-AGA TGT CCA GCC AGC TGC ACC TGA C-3′ and 5′-AGC CTC CGT TAT CCT GGA TCC A-3′ for *Bcl-2* primers (242 bp), and 5′-CAA TAT GAT TCC ACC CAT GGC AAA TTC CAT GGC AC-3′ and 5′-TGA AGT CAG AGG AGA CCA CCT GGT GCT

CAG TGT AG-3′ for *GAPDH* primers (718 bp). The RT-PCR (Onestep RT-PCR Kit) (Qiagen, Hilden, Germany) for *p53*, *p21*, *Bax*, *Bcl-2* and *GAPDH* mRNAs was performed according to the protocol described by Pecere et al. [32] and Usui et al. [33] with minor modification. The levels of these mRNAs were quantified from their band density on agarose gels using NIH Image software (National Institutes of Health, NJ, USA), and the ratio of the expression levels of *p53*, *p21*, *Bcl-2* and *Bax* mRNAs normalized relative to that of *GAPDH* mRNA. The RT-PCR kinetic curves of the ratios of the levels of these apoptosis-related signals mRNAs/*GAPDH* mRNA in this study could be determined quantitatively at least up to a ratio of approximately 5. Therefore, we could estimate the levels of *p53*, *p21*, *Bcl-2* and *Bax* mRNA expression quantitatively.

References

[1] M.M. Gattensman, I. Pasten, Annu. Rev. Biochem. 62 (1993) 385–427.
[2] O. Fardel, V. Lecurer, A. Guillonzo, Gen. Pharmacol. 27 (1996) 1283–1291.
[3] M.M. Gottensman, T. Fojo, S.E. Bates, Nat. Rev. Cancer 2 (2002) 48–58.
[4] R. Krishna, L.D. Mayer, Eur. J. Pharm. Sci. 11 (2000) 265–283.
[5] D.R. Ferry, H. Traunecker, D.J. Kerr, Eur. J. Cancer 32A (1996) 1070–1081.
[6] T. Tsukamoto, Y. Ueno, J. Pharm, Soc. Jpn. 56 (1936) 802–807.
[7] R.E. Marker, D.L. Turner, P.R. Ulshafer, J. Am. Chem. Soc. 62 (1940) 2542–2547.
[8] H.-J. Quan, J. Koyanagi, K. Ohmori, S. Uesato, T. Tsuchido, S. Saito, Eur. J. Med. Chem. 37 (2002) 659–669.
[9] C. Corbiere, B. Liagre, A. Bianchi, K. Bordji, M. Dauca, P. Netter, et al., Int. J. Oncol. 22 (2003) 899–905.
[10] M. Morisaki, N. Koizumi, N. Ikekawa, T. Takeshita, S. Ishimoto, J. Chem. Soc. Perkin Trans. I (1975) 1421–1424.
[11] N. Kubodera, K. Miyamoto, M. Matsumoto, T. Kawanishi, H. Ohkawa, T. Mori, Chem. Pharm. Bull. (Tokyo) 40 (1992) 648–651.
[12] B. Schönecker, M. Reichenbächer, S. Gliesing, M. Gonschior, S. Griebenow, D. Scheddin, et al., Steroids 63 (1998) 28–36.
[13] A.B. Turner, J. Chem. Soc. (1968) 2568–2570 C.
[14] D.H.R. Barton, R.H. Hesse, M.M. Pechet, E. Rizzardo, J. Am. Chem. Soc. 95 (1973) 2748–2749.
[15] S.C. Eyley, D.H. Williams, J. Chem. Soc. Chem. Commun. (1975) 858.
[16] M. Morisaki, A. Saika, K. Bannai, M. Sawamura, J. Rubio-Lightbourn, N. Ikekawa, Chem. Pharm. Bull. (Tokyo) 23 (1975) 3272–3278.
[17] A.W. Norman, X. Song, L. Zanello, C. Bula, W.H. Okamura, Steroids 64 (1999) 120–128.
[18] M. Liu, B. Yu, X. Wu, Y. Hui, K.-P. Fung, Carbohydr. Res. 329 (2000) 745–754.
[19] E. Havinga, Experientia 29 (1973) 1181–1193.
[20] Y. Yoshida, Y. Sakakura, N. Aso, S. Okada, Y. Tanabe, Tetrahedron 55 (1999) 2183–2192.
[21] F. De Simone, A. Dini, E. Finamore, L. Minale, C. Pizza, R. Riccio, et al., J. Chem. Soc. Perkin I (1981) 1855–1862.
[22] M. Shionoya, T. Jimbo, M. Kitagawa, T. Soga, A. Tohgo, Cancer Sci. 94 (2003) 459–466.
[23] G. Lee, M. Piqette-Miller, Can. J. Physiol. Pharmacol. 79 (2001) 876–884.
[24] K. Takara, T. Sakaeda, Y. Tanigawara, K. Nishiguchi, N. Ohmoto, M. Horinouchi, et al., Eur. J. Pharm. Sci. 16 (2002) 159–165.
[25] T. Mosmann, J. Immunol. Methods 139 (1983) 55–63.
[26] C.M. Chresta, J.R. Masters, J.A. Hickman, Cancer Res. 56 (1996) 1834–1841.
[27] T. Strobel, L. Swanson, S. Korsmeyer, S.A. Cannistra, Proc. Natl. Acad. Sci. USA 93 (1996) 14094–14099.
[28] H. Sawa, T. Kobayashi, K. Mukai, W. Zhang, H. Shiku, Int. J. Oncol. 16 (2000) 745–749.
[29] M. Takahashi, J. Kigawa, Y. Minagawa, H. Itamochi, M. Shimada, S. Samazawa, et al., Eur. J. Cancer 36 (2000) 1863–1868.
[30] P. Perego, M. Giarola, S.C. Righetti, R. Supino, C. Caserini, D. Delia, et al., Cancer Res. 56 (1996) 556–562.
[31] G.-Z. Jin, H.-J. Quan, J. Koyanagi, K. Takeuchi, Y. Miura, F. Komada, et al., Cancer Lett. 218 (2005) 15–20.
[32] T. Pecere, F. Sarinella, C. Salata, B. Gatto, A. Bet, F.D. Vecchia, A. Diaspro, M. Carli, M. Palumbo, G. Palu, Int. J. Cancer 106 (2003) 836–847.
[33] T. Usui, Y. Saitoh, F. Komada, Biol. Pharm. Bull. 26 (2003) 510–517.