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Original article

Preparations of heterospirostanols and their pharmacological activities

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Abstract

(3β,20*S*,22*S*,25*R*)-22-Thiospirosol-5-en-3-ol (**9**) and (3β,20*S*,22*S*,25*R*)-22-seleno-spirosol-5-en-3-ol (**11**) were prepared from diosgenin (**3**) via 26-iodopseudodiosgenin (**6**) as a key intermediate. Diosgenone (**15**), solasodinone (**16**), (20*S*,22*S*,25*R*)-22-thiospirosol-4-en-3-one (**17**), (20*S*,22*S*,25*R*)-22-selenospirosol-4-en-3-one (**18**) and (20*R*,22*S*,25*R*)-spirosol-4-en-3-one (**19**) were prepared by Oppenauer oxidation of **3**, solasodine **4**, **9**, **11** and (3β,20*R*,22*R*,25*R*)-spirosol-5-en-3-ol **14**, respectively. Oxidations of **15** and **16** with 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) provided corresponding dienone products, (20*S*,22*S*,25*R*)-spirosol-1,4-dien-3-one (**20**) and (20*S*,22*S*,25*R*)-22-thiospirosol-1,4-dien-3-one (**21**), respectively, while oxidation of **19** (C-20 diastereoisomer of **15**) gave no dienone product but 21-exo vinyl product **22.** 26-Thioacetylpseudodiosgenone (**24**) and 26-cyanoselenopseudodiosgenone (**25**) were prepared by treatment of 26-iodopseudodiosgenose (**23**), which was obtained by Oppenauer oxidation of **6**, with potassium thioacetate and potassium selenocyanate, respectively. Compounds **15** and **19** exhibited more than 80% inhibitions in INF-γ productions at 10.0 μM. Compounds **4** and **25** showed cytotoxic activities ($IC_{50} = 6$ and 5 μM, respectively) against cancerous HCT 116 cell lines. Compounds **12** and **25** had antiurease activities ($IC_{50} = 12.4$ and 11.4 μM, respectively), in which only the latter showed an inhibition zone (mean zone diameter = 12.2 mm) formed by *Bacillus subtilis* 168 *trp*.

Keywords: Heterospirostanol; Interferon-γ production inhibition; Cytotoxic activity; Antiurease activity; Antibacterial activity

1. Introduction

It has been reported that saponins have various pharmacological activities such as an antitumor activity [1], an antiallergic activity [2], an anti-inflammatory activity [3] an anti-HIV activity [4], and etc. Previously, we reported the structure–activity relationships on anti-hepatitis [5–11] and anti-HIV activities [12] of several glycosides having various aglycons derived from glycyrrhetic acid [13]. Niwa et al. [14] reported that timosaponin A-III (**1**) (spirostanol glycoside) had anti-platelet agglutination activity. We found that the anti-platelet agglutination activity largely depended on the sterochemistry of the E and F rings of spirostanols [15]. Tomatine (**2**) [16], which is the one of spirostanol analogues having a nitrogen atom instead of an oxygen atom on the F ring, showed an antibiotic activity [17] which was not observed in **1** (Fig. 1). The differences in the activity between **1** and **2** led us to attempt to prepare spirostanol derivatives having other hetero atoms such as sulfur and selenium on the F ring. The present study deals with preparations of various heterospirostanol derivatives having sulfur and selenium atoms as well as oxygen and nitrogen atoms on the F ring from diosgenin (**3**). Furthermore, pharmacological activities are further investigated for the inhibition of interferon-γ (IFN-γ) production, cytotoxic activities, antiurease activities and antibacterial activities of the derivatives.

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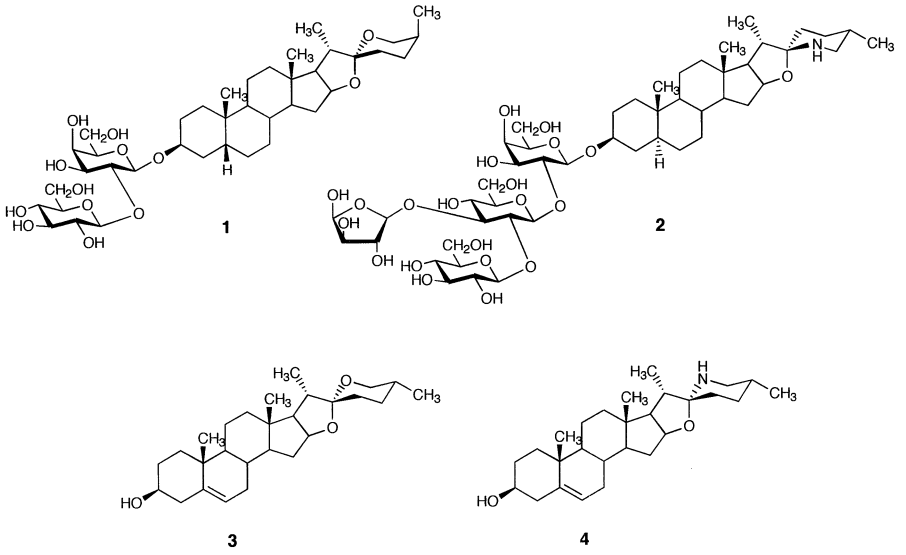

Fig. 1. Structures of compounds **1**–**4**.

2. Chemical results and discussion

Uhle [18] reported on the preparation of solasodine **4**, comprising a nitrogen atom on the F ring, by the hydrolysis of 26-phthalimidopseudodiosgenin (**5**) derived from 26-iodopseudodiosgenin (**6**) (Fig. 2). We planed to synthesise some spirostanol derivatives having a sulfur or a selenium atom on the F ring using **6** as a key intermediate.

Compound **6** was reacted with potassium thioacetate to give 26-thioacetyl-pseudodiosgenin (**7**) in 71% yield. Compound **8** obtained by alkaline hydrolysis of **7** showed a quasimolecular ion peak $[M + Na]^+$ at m/z 881 in the fast-atom-bombardment mass (FABMS) spectrum, which suggests that compound **8** is a disulphide dimer. The treatment of **8** with zinc powder in hot acetic acid [19], followed by alkaline hydrolysis, gave compound **9** in 71% yield, which showed an ion peak $[M + Na]^+$ at m/z 453 in the FABMS spectrum. It was thus deduced that **9** should be one of the four isomers, **9a** (normal F ring, 21α-CH_3), **9b** (normal F ring, 21β-CH_3), **9c** (iso F ring, 21α-CH_3) and **9d** (iso F ring, 21β-CH_3), with respect to the conformation of the F ring and the configuration at C-20. These isomers were products to be obtainable from **7** by alkaline hydrolysis followed by cyclization of the side chain at C-22 to form the spiro F ring (Figs. 1–3). The ^{1}H-NMR spectrum of **9** was compared with that of 3-*O*-benzy-(3β,20*S*,22*S*,25*R*)-22-thiospirosol-5-en-3-ol **10** whose absolute structure was determined by the X-ray analysis [20]. The proton signals of **9** were superimposable to those of **10** except for the signals due to the substituent at *O*-3 (see Experimental), which suggests that **9** is (3β,20*S*,22*S*,25*R*)-22-thiospirol-5-en-3-ol (**9a**). Production of sole cyclization compound **9a** in the sequential alkaline hydrolysis of **7** and the ring closure of the resulting intermediate by treatment with acetic acid was consistent with the previous report that **9a** was the most thermodynamically stable among the four obtainable isomers **9a**–**9d** [15].

According to the foregoing methods, (3β,20*S*, 22*S*,25*R*)-22-selenospirosol-5-en-3-ol (**11**) was prepared from 26-iodo intermediate **6**. Compound **6** was reacted with potassium selenocynate [21] to give 26-selenocyanopseudodiosgenin **12** (showed a peak of $[M + Na]^+$ at m/z 526 in the FABMS spectrum) in 71% yield. Compound **12** was hydrolysed by potassium carbonate [21] under argon atmosphere, followed by a treatment with acetic acid, to provide white powdered product **13**. Compound **13** showed a peak $[M + Na]^+$ at m/z 977 in FABMS spectrum, which suggests that **13** is 26,26′-(bispseudodiosgeninyl) diselenide. Compound **13** was treated with zinc powder in hot acetic acid, followed by alkaline hydrolysis, to provide **11** in 78% yield, which showed an ion peak $[M + Na]^+$ at m/z 501 in FABMS spectrum. The conformation of the F ring and the configuration at C-20 of **11** were determined by comparison of its ^{1}H- and ^{13}C-NMR spectra with those of **9**. The proton signals of 26-H's, 18-CH_3, 21-CH_3 and

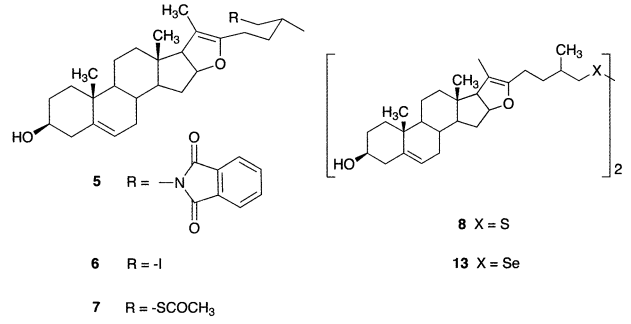

Fig. 2. Structures of compounds **5**–**8** and **13**.

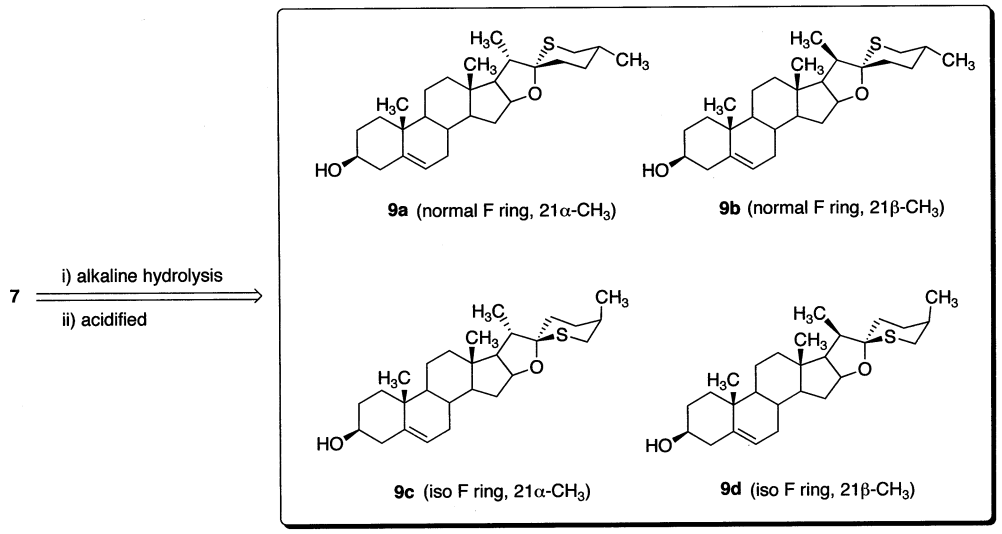

Fig. 3. Possible structures of compound **9** obtained from **7** by alkaline hydrolysis followed by acidification.

Table 1
^{13}C-NMR spectral data of compounds **9**, **11**, **17**–**19** and **21**–**22** [a]

	9	11	17	18	19	21	22
C-1	37.2 [b]	37.2	35.6	35.7	35.7	155.7	37.2
C-2	31.4	31.1	33.9	33.9	33.9	127.5	31.5
C-3	71.7	71.6	199.4	199.5	199.5	186.2	71.5
C-4	42.2	42.2	123.9	123.9	123.9	123.9	42.2
C-5	140.8	140.8	171.0	171.0	171.1	168.9	140.9
C-6	121.4	121.3	32.8	32.8	32.8	32.7	121.2
C-7	31.6	31.5	31.3	31.0	31.9	33.7	31.9
C-8	33.3	34.0	35.2	34.0	35.0	35.1	31.8
C-9	50.0	50.0	53.7	53.7	53.8	52.4	50.0
C-10	36.6	36.6	38.5	38.6	38.6	43.5	36.6
C-11	20.8	20.8	20.8	20.8	20.4	22.7	20.5
C-12	39.7	39.7	39.6	39.6	39.6	39.5	38.6
C-13	40.3	40.4	40.4	40.5	41.8	40.7	42.5
C-14	56.6	56.6	55.7	55.7	56.7	55.3	56.8
C-15	31.7	32.0	31.7	32.1	30.6	31.5	31.1
C-16	81.6	82.7	81.4	82.5	80.8	81.3	81.1
C-17	62.8	62.9	62.7	62.9	60.5	62.7	59.5
C-18	16.6	16.6	16.6	16.7	16.1	16.7	14.6
C-19	19.4	19.4	17.4	17.4	17.4	18.8	19.4
C-20	44.4	45.4	44.4	45.5	46.4	44.4	150.0
C-21	16.2	17.7	16.2	17.7	11.3	16.1	107.8
C-22	97.5	97.9	97.4	97.8	108.3	97.4	106.9
C-23	38.5	40.1	38.6	40.1	32.0	38.5	32.6
C-24	32.0	32.6	32.1	32.7	28.5	31.7	28.6
C-25	31.4	31.4	33.2	35.2	30.6	33.2	30.3
C-26	32.1	24.8	32.1	24.9	68.1	32.1	67.6
C-27	22.5	23.7	22.5	23.7	17.2	22.5	17.2

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

27-CH_3 of **11** were observed at the fields similar to those of the corresponding proton signals of **9** (see Section 2). Furthermore, the carbon signals at C-18, C-20, C-21, C-22 and C-27 of **11** were observed at similar fields to those of the corresponding carbon signals of **9** (see Table 1), suggesting that **11** is (3β,20*S*,22*S*,25*R*)-22-selenospirosol-5-en-3-ol (Fig. 4).

Oppenauer oxidations [22,23] of compounds **3**, **4**, **9**, **11** and **14** [15] gave corresponding α,β-unsaturated ketone derivatives **15** [22], **16** [23], **17**, **18** and **19** in 79,

68, 81, 74 and 37% yields, respectively. Oxidations of **15**–**19** with 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) were performed in order to obtain the corresponding dienone derivatives. Treatments of **15** and **17** with DDQ gave the corresponding dienone derivatives **20** [24] and **21** in 49 and 21% yields, respectively. However, the same treatments of **16** and **18** gave unisolable products. Interestingly, the oxidation of **19** with DDQ gave a new olefinic product **22**, though in a low yield (23% yield), together with unisolable products but no dienone derivative. As we are also interested in pharmacological activities of pseudodiosgenone derivatives having an α,β-unsaturated ketone on the A ring, Oppenauer oxidations of **7** and **12** were attempted. However, these resulted in failure to obtain desired 26-thioacetyl-pseudodiosgenone (**24**) and 26-cyanoselenopseudodiosgenone (**25**). It may be attributable to alkaline which has a high sensitivity to a thioacetyl group of **7** or to a cyanoseleno group of **12**. Compounds **24** and **25** were finally obtained as follows; the reactions of **23**, which was derived by Oppenauer oxidation of **6**, with potassium thioacetate and potassium selenocyanate in 73 and 74% yields, respectively.

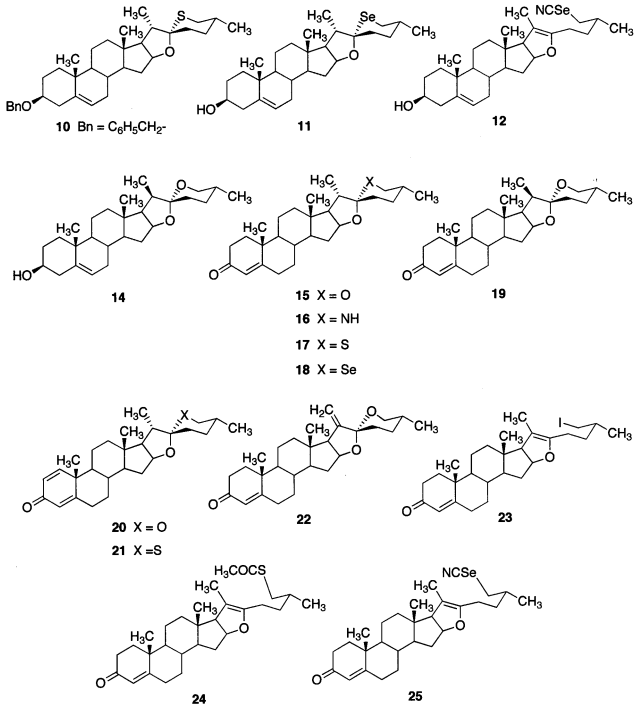

Fig. 4. Structures of compounds **10**–**12** and **14**–**25**.

Table 2
Inhibitory activities of INF-γ production from $CD4^+$ T cells stimulated with anti-CDS and anti-CD28

Compounds	Concentration (μM)	Inhibition (%)
15	1.0	18.1
15	3.0	48.6
15	10.0	80.0
16	10.0	71.7
17	10.0	31.0
18	10.0	33.5
19	10.0	81.2
20	10.0	50.0
21	10.0	47.6
22	10.0	68.7

3. Pharmacological results and discussion

Inhibitory activities in INF-γ production, cytotoxic activities of cancerous HCT 116 cells and antiurease activities as well as antibacterial activities were investigated using steroidal compounds obtained in this study.

Inhibitory activities of spiro compounds **3** and **14**–**22** against the INF-γ productions were measured by the sandwicth ELISA method [25] using stimulated BALB/c splenic T cells [26] according to the method by Zhang et al. [27] The assays for of all the compounds were performed at the concentrations of 1.0, 3.0 and 10.0 μM as shown in the compound **15** in Table 2. In Table 2, compounds **15**–**22** showed the inhibition of more than 30% except for compound **15** at the concentration of 1.0 μM. Diosgenin **3** and its C-20 isomer **14** showed the inhibition lower than 30% even at the concentration of 10.0 μM. However, in spiro derivatives **15**–**19** and **22**, which have an α,β-unsaturated ketone group in the A rings, **15**, **19** and **22** having an oxygen atom and **16** having a nitrogen atom on the F rings exhibited efficiencies (more than ca. 70% inhibition) while **17** and **18** having sulfur and selen atoms showed lower activities (31.0 and 33.5%, respectively). The tendency for these activities may be due to the electronegativities of the hetero atoms. Thiospirostanol derivative **20** and selenospirostanol derivative **21** having a dienone structure in the A rings showed moderate activities (50.0 and 47.6%, respectively). These results indicate that it is essential to have an oxygen or a nitrogen in the F rings and an α,β-unsaturated ketone group in the A rings in the steroidal molecules to achieve high activity against INF-γ production inhibition.

In the investigation for cytotoxic effects against human cancer cell lines, compounds **3**, **4**, **9**, **11**, **15**–**18**, **20**, **21**, **24** and **25** were examined (Table 3). The assays on these compounds were conducted according to the method reported by Takenouchi and Munekata [28]. IC_{50} values were calculated based on the percentage inhibitions of the compounds against human colorectal HCT 116 carcinoma cells. Among the spirostanol derivatives **3**, **4**, **9** and **11**, which possess no conjugated system in the A rings but the hetero atoms, O, N, S and Se, respectively, **4** has efficient activity ($IC_{50} = 6$ μM). Thus, the nitrogen atom on the F ring seemed to be essential for the increment of the cytotoxic effect against HCT 116 cells. A similar tendency was observed

Table 3
Cytotoxic effects of compounds **3**, **4**, **9**, **11**, **15**–**18**, **20**, **21**, **24** and **25** against HCT 116 cells

Compounds	IC_{50} (μM)
3	>100
4	6
9	64
11	73
15	>100
16	14
17	>100
18	37
20	52
21	31
24	26
25	5

Table 4
Antiurease activities of compounds **12**, **16**, **24** and **25**

Compounds	IC_{50} (μM)
12	12.4
16	None
24	None
25	11.4

in spirosteroidal compounds **15**–**18** which had an α,β-unsaturated ketone system in the A ring and had the hetero atoms, O, N, S and Se, respectively. Specifically, **16** having a nitrogen atom on the F ring showed the highest activity ($IC_{50} = 14$ μM) among **15**–**18** although it was lower than that of **4** ($IC_{50} = 6$ μM). O- and S-spirostanols **20** and **21** having a dienone structure in the A ring had activities ($IC_{50} = 52$ and 31 μM, respectively) higher than activities of the corresponding spirostanols **3** and **9** having no conjugated system and those of **15** and **17** having an α,β-unsaturated ketone group, though they had activities lower than those of **4** and **16.** Interestingly, pseudo derivatives **24** and **25** showed activities of $IC_{50} = 26$ and 5 μM, respectively. Especially **25** having a SeCN group at C-26 exhibited almost the same efficiency as that of **4**.

For antiurease activities, only compounds **12**, **16**, **24** and **25** were investigated because the other compounds were poorly soluble in dimethyl sulfoxide. Pseudo compounds **12** and **25** having a SeCN group at C-26 showed the efficient antiurease activities ($IC_{50} = 12.4$ and 11.4 μM, respectively), but the others did not show such antiurease activities (Table 4), indicating that the SeCN substituent in the molecules may be contributed to the antiurease activity.

Antibacterial activities of steroidal compounds **3**, **4**, **8**, **9**, **11**–**18**, **20**, **21**, **24** and **25** were measured by the injury evaluation on bacterial cells of *Bacillus subtilis* 168 UV [29] using an image analysis of the cell motion [30,31]. In these compounds, **25** showed an antibacterial efficiency having a relatively low motion speed of 7.7 μm s^{-1} compared with control (without steroidal compound; 35.9 μm s^{-1}) while the other compounds showed no remarkable anti bacterial activity (28.3–34.6 μm s^{-1}). Furthermore, *B. subtilis* 168 *trp* [29] and *Escherichia coli* RP 470 [32] were employed for a disk assay. In the steroidal compounds, only compound **25** showed an inhibition zone (mean zone diameter = 12.2 mm) formed by *B. subtilis* 168 *trp*, but no inhibition zone formed by *E. coli* RP 470 was observed.

4. Conclusions

Heterospirostanols **9** and **11** which bear S atom and Se atom, respectively, on the F rings were synthesised from diosgenie **3**. Oppenarer oxidations of **3**, **4**, **9**, **11** and **14** (a C-20 isomer of **3**) gave corresponding α,β-unsaturated ketone **15**–**19**, respectively.

In the oxidations of **15**–**19** with DDQ, compounds **15** and **17** gave corresponding dienone derivatives **20** and **21**, and compound **19** gave a new olefinic product **22**. Pseudo derivatives **24** and **25**, which had an acetylthio group and a cyanoseleno group at C-26, were obtained from 26-iodopseudodiosgenin (**23**).

Several pharmacological activities were investigated using the heterospirostanol derivatives and the pseudo derivatives obtained in this study. The inhibitory activities of compounds **3** and **14**–**22** against INF-γ production were compared. Among these compounds, α,β-unsaturated ketone derivatives **15**, **19** and **22**, which have an oxygen atom, and **16** which has a nitorgen atom on the F rings exhibited efficiencies (more than ca. 70% inhibition) (Table 2). O- **20** and S-Spirostanol **21** having an oxygen and a sulfur atom, respectively, on the F-rings showed moderate activities (50.0 and 47.6%, respectively). These results indicate that for appearance of efficiency against INF-γ production inhibition, it is essential to have an oxygen or a nitrogen on the F rings and an α,β-unsaturated ketone group in the A rings.

Cytotoxic activities of spirostanol derivatives **3**, **4**, **9**, **11**, **15**–**18**, **20** and **21** and pseudo derivatives **24** and **25** against cancerous HCT 116 cells were evaluated. In the spirostanol derivatives, **4** and **16** exhibited efficiencies ($IC_{50} = 6$ and 14 μM, respectively) while the others exhibited low activities. It was thus suggested that the presence of a nitrogen atom on the F rings is essential to have efficiency against HCT 116 cells. Furthermore, N-spirostanols having no conjugated system in the A rings exhibited activities higher than those having a conjugated system. Interestingly, pseudo derivatives **24** and **25** showed activities of $IC_{50} = 26$ and 5 μM, respectively. Especially, **25** having a cyanoseleno group at C-26 showed almost the same efficiency as that of **4**.

The introduction of a cyanoseleno group to the pseudo derivatives provided antiurease and antibacterial activities. Specifically, pseudo derivatives **12** and **25** having the substituent at C-26 showed efficient antiurease activities ($IC_{50} = 12.4$ and 11.4 μM, respectively), and **25** also showed efficient antibacterial activity. These results may give us further attempts to investigate synthesis of new compounds having a cyanoseleno group in the molecules.

5. Experimental

5.1. General procedures

Diosgenin used herein was purchased from SIGMA Chemical Co. Ltd., USA. The other chemicals and solvents were of reagent grade and obtained from suppliers commercially available. Melting points (m.p.) were determined using a Yanagimoto micromelting point apparatus and were uncorrected. Kieselgel 60 F_{254} (E. Merck) was utilized for the thin-layer chromatography (TLC). Spots were detected by spraying 1:9 $Ce(SO_4)_2$–10% H_2SO_4 reagent, followed by heating the plate at 100 °C for 10 min. Column chromatographies were carried out using a Wacogel C-200. Then, the eluates were monitored using TLC. An SSC-6300 HPLC instrument (Senshu Scientific Co. Ltd.) was employed for an analytical HPLC (DOCOSIL, 10 × 250 mm; flow rate, 1.0 mL min^{-1}; column temperature 40 °C). The SSC-6300 HPLC instrument was further equipped with an SSC autoinjector 6310 and an SSC fraction collector 6320 for a preparative HPLC (DOCOSIL, 10 × 250 mm; flow rate, 1.0 mL min^{-1}; column temperature 40 °C). ^1H and ^{13}C-NMR at 500 and 125 MHz, respectively, as well as ^1H–^1H and ^1H–^{13}C COSY, DEPT and HMBC spectra were obtained with a JEOL JNM-A500 FT-NMR spectrometer. Tetramethylsilane was used as an internal standard. Chemical shifts were given in ppm. Multiplicities of ^1H-NMR signals were indicated as s (singlet), d (doublet), dd (doublet of doublets), t (triplet) and m (multiplet). FABMS and high resolution mass (HRMS) spectra were recorded on a JEOL LMS-DX 300 mass spectrometer.

5.1.1. Chemistry

*5.1.1.1. 26-Thioacetylpseudodiosgenin (**7**).* Potassium thioacetate (705 mg, 6.2 mmol) was added to a solution of **6** [18] (2.93 g, 5.5 mmol) in absolute acetone (200 mL). The mixture was stirred for 24 h at room temperature (r.t.). The reaction mixture was poured into ice-cold water (300 mL), and extracted with dichloromethane (200 mL × 3). The combined extracts were washed with brine, dried over anhydrous magnesium sulfate, and filtered. The filtrate was evaporated to obtain a residue that was subjected to a preparative HPLC (15% H_2O–acetone) to give **7** (1.86 g: yield 71%) as crystalline needles; m.p. 92–94 °C (after recrystallization from ethanol): FABMS: m/z 495 $[M + Na]^+$; ^1H-NMR ($CDCl_3$) (only assignable signals were listed) δ 5.34 (1H, d, $J = 5.2$ Hz, H-6), 4.73 (1H, ddd, $J =$ 10.1, 7.9, 5.8 Hz, H-16), 3.52 (1H, m, H-3), 2.93 (1H, dd, $J = 13.1$, 5.5 Hz, H-26a), 2.77 (1H, dd, $J = 13.1$, 7.3 Hz, H-26b), 2.46 (1H, d, $J = 10.1$ Hz, H-17), 2.32 (3H, s, $SCOCH_3$), 1.58 (3H, s, 21-CH_3), 1.02 (3H, s, 19-CH_3), 0.94 (3H, d, $J = 6.7$ Hz, 27-CH_3), 0.68 (3H, s, 18-CH_3); ^{13}C-NMR ($CDCl_3$) δ 195.9 ($SCOCH_3$), 151.4 (C-22), 140.8 (C-5), 121.3 (C-6), 103.9 (C-20), 84.3 (C-16), 71.6 (C-3), 64.2 (C-17), 55.0 (C-14), 50.1 (C-9), 43.2 (C-13), 42.2 (C-4), 39.5 (C-1), 37.2 (C-12), 36.6 (C-10), 35.7 (C-26), 34.1 (C-15), 33.3 (C-24), 32.8 (C-25), 32.2 (C-7), 31.6 (C-2), 31.2 (C-8), 30.7 ($SCOCH_3$), 23.3 (C-11), 21.0 (C-23), 19.4 (C-19), 19.0 (C-27), 13.9 (C-18), 11.6 (C-21); HRMS (FAB) m/z calc. for $C_{29}H_{44}NaO_3S$ $[M + Na]^+$, 495.2909. Found: 495.2811%.

*5.1.1.2. 26,26′-(Bispseudodiosgeninyl) disulfide (**8**).* A solution of **7** (0.90 g, 1.9 mmol) in 0.2 M-NaOH (H_2O–EtOH = 1/9) (200 mL) was stirred for 24 h under argon atmosphere at r.t. The reaction mixture was poured into acetic acid. The resulting mixture was poured into ice-cold water (300 mL) and then extracted with dichloromethane (200 mL × 3). The combined extracts were washed with 5% $NaHCO_3$ aqueous solution and brine, dried over anhydrous magnesium sulfate and filtered. The filtrate was evaporated to obtain a residue that was subjected to a preparative HPLC (5% H_2O–acetone) to give **8** (450 mg, 55% yield) as crystalline needles, m.p. 82–84 °C (after recrystallization from ethanol). FABMS: m/z 881 $[M + Na]^+$; ^1H-NMR ($CDCl_3$) (only assignable signals were listed) δ 5.35 (1H, d, $J = 5.2$ Hz, H-6), 4.73 (1H, ddd, $J = 10.1$, 7.6, 5.5 Hz, H-16), 3.52 (1H, m, H-3), 2.75 (1H, dd, $J =$ 12.8, 5.5 Hz, H-26a), 2.51 (1H, dd, $J = 12.8$, 7.9 Hz, H-26b), 2.47 (1H, d, $J = 10.1$ Hz, H-17), 1.59 (3H, s, 21-CH_3), 1.02 (3H, s, 19-CH_3), 1.00 (3H, d, $J = 6.7$ Hz, 27-CH_3), 0.69 (3H, s, 18-CH_3); ^{13}C-NMR ($CDCl_3$) δ 151.5 (C-22), 140.8 (C-5), 121.4 (C-6), 103.8 (C-20), 84.3 (C-16), 71.7 (C-3), 64.2 (C-17), 55.0 (C-14), 50.1 (C-9), 46.8 (C-26), 43.3 (C-13), 42.3 (C-4), 39.5 (C-1), 37.3 (C-12), 36.6 (C-10), 34.1 (C-15), 33.4 (C-24), 32.6 (C-25), 32.2 (C-7), 31.6 (C-2), 31.3 (C-8), 23.4 (C-11), 21.0 (C-23), 19.4 (C-19), 18.9 (C-27), 14.0 (C-18), 11.7 (C-21); HRMS (FAB) m/z calc. for $C_{54}H_{82}NaO_4S_2$ $[M + Na]^+$, 881.5552. Found: 881.5538%.

*5.1.1.3. (3β,20S,22S,25R)-22-Thiospirosol-5-en-3-ol (**9**).* To a solution of **8** (70 mg, 0.08 mmol) in acetic acid (10 mL), zinc powder (20 mg, 0.31 mmol) was added. The

mixture was refluxed for 24 h and filtered. The filtrate was co-evaporated with toluene under reduced pressure to give a residue. The residue was dissolved in acetone (1 mL) and added with a 10% KOH solution (H_2O–EtOH = 1/1) (5 mL), then the mixture was stirred for 24 h at r.t. The mixture was poured into ice-cold water (40 mL) and extracted with dichloromethane (70 mL × 3). The extracts were washed with brine, dried over anhydrous magnesium sulfate and then filled. The filtrate was evaporated and the resulting residue was purified by a preparative HPLC (15% H_2O–acetone) to give **9** (50 mg, yield 71%) as crystalline needles, m.p. 212–214 °C (after recrystallized from ethanol): FABMS: m/z 453 $[M + Na]^+$; ^{1}H-NMR ($CDCl_3$) (only assignable signals were listed) δ 5.33 (1H, d, $J = 5.2$ Hz, H-6), 4.61 (1H, dd, $J = 15.3$, 7.6 Hz, H-16), 3.50 (1H, m, H-3), 2.52 (1H, dd, $J = 13.1$, 11.6 Hz, H-26a), 2.26 (1H, dd, $J = 13.1$, 2.1 Hz, H-26b), 1.01 (3H, s, 19-CH_3), 1.00 (3H, d, $J = 7.3$ Hz, 21-CH_3), 0.91 (3H, d, $J = 6.7$ Hz, 27-CH_3), 0.82 (3H, s, 18-CH_3); ^{13}C-NMR spectral data were listed in Table 1; HRMS (FAB) m/z calc. for $C_{27}H_{43}O_3S$ $[M + H]^+$, 431.2983. Found: 431.2968.

*5.1.1.4. 3-O-Benzyl-(3β,20S,22S,25R)-22-thiospirosol-5-ene (**10**).* To a suspension of 3-*O*-benzyl-26-thioacetylpseudodiosgenin (1.0 g, 1.8 mmol) in MeOH (200 mL), 10% HCl in MeOH (200 mL) was added. The mixture was refluxed for 36 h and evaporated under reduced pressure to give a residue. The residue was added with ice-cold water (300 mL), and extracted with dichloromethane (200 mL × 3).

The combained extracts were washed with brine, dried over anhydrous magnesium sulfate and filtered. The filtrate was evaporated to obtain a residue that was purified by column chromatography (a gradient of 0–2% ethyl acetate in benzene) to obtain **10** (544 mg: yield 56%) as needles, m.p. 149–151 °C (after recrystallization from ethanol): FABMS: m/z 543 $[M + Na]^+$; ^{1}H-NMR ($CDCl_3$) (only assignable signals were listed) δ 7.35–7.24 (5H, aromatic protons), 5.34 (1H, d, $J = 5.2$ Hz, H-6), 4.63 (1H, dd, $J = 15.3$, 7.6 Hz, H-16), 4.55 (2H, d, $J = 2.1$ Hz, $C_6H_5CH_2$), 3.27 (1H, m, H-3), 2.53 (1H, dd, $J = 12.8$, 11.6 Hz, H-26a), 2.28 (1H, dd, $J = 12.8$, 1.8 Hz, H-26b), 1.03 (3H, s, 19-CH_3), 1.01 (3H, d, $J = 7.0$ Hz, 21-CH_3), 0.92 (3H, d, $J = 6.4$ Hz, 27-CH_3), 0.80 (3H, s, 18-CH_3); ^{13}C-NMR ($CDCl_3$) δ 140.9 (C-5), 138.9 ($C_6H_5CH_2$), 128.2 ($C_6H_5CH_2 \times 2$), 127.4 ($C_6H_5CH_2 \times 2$), 127.3 ($C_6H_5CH_2$), 121.2 (C-6), 97.4 (C-22), 81.5 (C-16), 78.4 (C-3), 69.8 ($C_6H_5CH_2$), 62.7 (C-17), 56.5 (C-14), 50.0 (C-9), 44.3 (C-20), 40.2 (C-13), 39.7 (C-12), 39.0 (C-4), 38.5 (C-23), 37.1 (C-1), 36.9 (C-10), 33.2 (C-8), 32.0 (C-24, C-26), 31.7 (C-15), 31.3 (C-7, C-25), 28.3 (C-2), 22.4 (C-27), 20.7 (C-11), 19.3 (C-19), 16.4 (C-18), 14.3 (C-21); HRMS (FAB) m/z calc. for $C_{34}H_{49}O_3S$ $[M + H]^+$, 521.3453. Found: 521.3427.

*5.1.1.5. 26-Cyanoselenopseudodiosgenin (**12**).* To a solution of **6** (1.45 g, 2.8 mmol) in DMF (130 mL), potassium selenocyanate (2.0 g, 13.9 mmol) was added. The mixture was stirred for 24 h at 60 °C, and then poured into ice-cold water (150 mL). The mixture was extracted with dichloromethane (150 mL × 3). The extracts were washed with 5% $NaHCO_3$ aqueous solution and brine, dried over anhydrous magnesium sulfate and filtered. The filtrate was evaporated to obtain a residue that was subjected to a preparative HPLC (15% H_2O–acetone) to give **12** (0.98 g, 71% yield) as needles, m.p. 106–108 °C (after recrystallization from ethanol): FABMS: m/z 526 $[M + Na]^+$; ^{1}H-NMR ($CDCl_3$) (only assignable signals were listed) δ 5.35 (1H, d, $J = 5.5$ Hz, H-6), 4.75 (1H, ddd, $J = 10.1$, 7.6, 5.5 Hz, H-16), 3.52 (1H, m, H-3), 3.16 (1H, dd, $J = 11.9$, 5.2 Hz, H-26a), 2.95 (1H, dd, $J = 11.9$,7.3 Hz, H-26b), 2.48 (1H, d, $J = 10.1$ Hz, H-17), 1.60 (3H, s, 21-CH_3), 1.07 (3H, d, $J = 6.7$ Hz, 27-CH_3), 1.02 (3H, s, 19-CH_3), 0.69 (3H, s, 18-CH_3); ^{13}C-NMR ($CDCl_3$) δ 150.6 (C-22), 140.8 (C-5), 121.3 (C-6), 104.4 (C-20), 102.0 (SeCN), 84.4 (C-16), 71.6 (C-3), 64.0 (C-17), 54.9 (C-14), 50.0 (C-9), 43.2 (C-13), 42.2 (C-4), 39.4 (C-1), 37.6 (C-26), 37.2 (C-12), 36.5 (C-10), 34.0 (C-15), 33.6 (C-25), 33.1 (C-24), 32.1, (C-7), 31.5 (C-2), 31.2 (C-8), 23.1 (C-11), 20.9 (C-23), 19.4 (C-19), 19.2 (C-27), 13.9 (C-18), 11.6 (C-21); HRMS (FAB) m/z calc. for $C_{28}H_{41}NNaO_2Se$ $[M + Na]^+$, 526.2200. Found: 526.2199.

*5.1.1.6. 26,26′-(Bispseudodiosgenyl) diselenide (**13**).* A solution of **12** (1.50 g, 3.0 mmol) and potassium carbonate (1.7 g) in DMF (70 mL) containing water (7.2 mL) was stirred for 8 h at 80 °C. The reaction mixtrue was acidified with acetic acid, poured into ice-cold water (250 mL), and then extracted with dichloromethane (250 mL × 3). The combined extracts were washed successively with 5% $NaHCO_3$ aqueous solution and brine, dried over anhydrous magnesium sulfate, and filtered. The filtrate was evaporated to give a residue that was recrystallized from EtOH–water to give **13** (0.91 g, 64% yield) as needles, m.p. 115–117 °C: FABMS: m/z 977 $[M + Na]^+$; ^{1}H-NMR ($CDCl_3$) (only assignable signals were listed) δ 5.34 (1 H, d, $J = 5.2$ Hz, H-6), 4.74 (1H, ddd, $J = 10.1$, 7.9, 5.8 Hz, H-16), 3.52 (1H, m, H-3), 3.03 (1H, dd, $J = 11.9$, 5.2 Hz, H-26a), 2.83 (1 H, dd, $J = 11.9$, 7.9 Hz, H-26b), 2.47 (1 H, d, $J = 10.1$ Hz, H-17), 1.59 (3H, s, 21-CH_3), 1.02 (3H, s, 19-CH_3), 1.01 (3H, d, $J = 6.7$ Hz, 27-CH_3), 0.69 (3H, s, 18-CH_3); ^{13}C-NMR ($CDCl_3$) δ 151.5 (C-22), 140.8 (C-5), 121.3 (C-6), 103.8 (C-20), 84.3 (C-16), 71.6 (C-3), 64.2 (C-17), 55.0 (C-14), 50.0 (C-9), 43.2 (C-13), 42.2 (C-4), 39.5 (C-1), 39.1 (C-26), 37.2 (C-12), 36.6 (C-10), 34.1 (C-15), 33.9 (C-25), 33.8 (C-24), 32.2 (C-7), 31.6 (C-2), 31.2 (C-8), 23.4 (C-11), 21.0 (C-23), 19.5 (C-27), 19.4 (C-19), 14.0 (C-18), 11.7 (C-21); HRMS (FAB) m/z calc. for $C_{54}H_{82}NaO_4Se_2$, $[M + Na]^+$, 977.4441. Found: 977.4509.

*5.1.1.7. (3β,20S,22S,25R)-22-Selenospirosol-5-en-3-ol (**11**).* A suspension of **13** (910 mg, 0.95 mmol) and zinc powder (180 mg, 2.75 mmol) in acetic acid (90 mL) was refluxed for 24 h. The mixture was filtered, and the filtrate was co-evaporated with toluene under reduced pressure to obtain a residue. The residue was dissolved in dioxane (60 mL), added with 10% KOH (H_2O–EtOH = 1/1) (50 mL), and then stirred for 2 h at r.t. The mixture was poured into ice-cold water (200 mL) and extracted with dichloromethane (200 mL × 3). The organic extracts were washed with brine, dried over anhydrous magnesium sulfate, and filtered. The filtrate was evaporated to obtain a residue that was recrystallized from EtOH–water to give **11** (0.71 g, 78% yield) as needles, m.p. 236–238 °C: FABMS: m/z 501 $[M + Na]^+$; ^{1}H-NMR($CDCl_3$) (only assignable signals were listed) δ 5.35 (1H, d, $J = 5.2$ Hz, H-6), 4.64 (1H, dd, $J = 15.6$, 7.6 Hz, H-16), 3.52 (1H, m, H-3), 2.59 (1H, t, $J = 11.9$ Hz, H-26a), 2.37 (1 H, d, $J = 11.9$ Hz, H-26b), 1.02 (3H, s, 19-CH_3), 1.01 (3H, d, $J = 6.7$ Hz, 21-CH_3), 0.97 (3H, d, $J = 6.7$ Hz, 27-CH_3), 0.82 (3H, s, 18-CH_3); ^{13}C-NMR spectral data were listed in Table 1; HRMS (FAB) m/z calc. for $C_{27}H_{42}NaO_2Se$ $[M + Na]^+$, 501.2248. Found: 501.2259%.

*5.1.1.8. (20S,22S,25R)-22-Thiospirosol-4-en-3-one (**17**).* A suspension of **9** (1.50 g, 3.5 mmol) and cyclohexanone (6.3 mL) in toluene (15 mL) was added with aluminum *tert*-butoxide (1.40 g, 5.7 mmol) in toluene (50 mL), and then refluxed for 11 h. The mixture was poured into ice-cold water (200 mL), and then added with 10% HCl until solid massed were dissolved. The reaction mixture was extracted with dichloromethane (100 mL × 3). The combined organic extracts were washed with 5% $NaHCO_3$ aqueous solution and brine, dried over anhydrous magnesium sulfate and filtered. The filtrate was evaporated to give a residue that was subjected to column chromatography (a gradient of 0–2% ethyl acetate in benzene) to obtain **17** (1.21 g, 81% yield) as neeldes, m.p. 213–215 °C (after recrystallization from ethanol): FABMS: m/z 451 $[M + Na]^+$; ^{1}H-NMR ($CDCl_3$) (only assignable signals were listed) δ 5.72 (1H, s, H-4), 4.63 (1H, dd, $J = 15.3$, 7.6 Hz, H-16), 2.52 (1H, t, $J = 11.9$ Hz, H-26a), 2.29 (1H, m, H-26b), 1.20 (3H, s, 19-CH_3), 1.01 (3H, d, $J = 7.0$ Hz, 21-CH_3), 0.93 (3H, d, $J = 6.4$ Hz, 27-CH_3), 0.84 (3H, s, 18-CH_3); ^{13}C-NMR spectral data were listed in Table 1; HRMS (FAB) m/z calc. for $C_{27}H_{41}O_2S$ $[M + H]^+$, 429.2819. Found: 429.2818%.

*5.1.1.9. (22S,25R)-22-Se-Spirosol-4-en-3-one (**18**).* The general procedure for Oppenauer oxidation was employed for **11** (710 mg, 1.5 mmol) to give a residue that was subjected to a preparative HPLC (5% H_2O–acetone) to obtain **18** (520 mg, 74% yield) as needles, m.p. 206–208 °C (after recrystallization from ethanol): FABMS: m/z 499 $[M + Na]^+$; ^{1}H-NMR ($CDCl_3$) (only assignable signals were listed) δ 5.73 (1H, s, H-4), 4.65 (1H, dd, $J = 15.3$, 7.6 Hz, H-16), 2.59 (1H, t, $J = 11.9$ Hz, H-26a), 2.37 (1H, dd, $J = 11.9$, 3.1 Hz, H-26b), 1.20 (3H, s, 19-CH_3), 1.03 (3H, d, $J = 6.7$ Hz, 21-CH_3), 0.97 (3H, d, $J = 6.7$ Hz, 27-CH_3), 0.84 (3H, s, 18-CH_3); ^{13}C-NMR spectral data were listed in Table 1; HRMS (FAB) m/z calc. for $C_{27}H_{41}O_2Se$ $[M + H]^+$, 477.2272. Found: 477.2260%.

*5.1.1.10. (20R,22S,25R)-spirosol-4-en-3-one (**19**).* The general procedure for Oppenauer oxidation was employed for **14** [15] (1.20 g, 2.9 mmole) to obtain a residue that was purified by column chromatography (a gradient of 0–5% ethyl acetate in benzene) to obtain **19** (440 mg, 37% yield) as needles, m.p. 166–168 °C (after recrystallization from ethanol): FABMS: m/z 435 $[M + Na]^+$; ^{1}H-NMR ($CDCl_3$) (only assignable signals were listed) δ 5.72 (1H, s, H-4), 4.45 (1H, dt, $J = 10.4$, 3.6 Hz, H-16), 3.48 (2H, m, H-26a, H-26b), 1.19 (3H, s, 19-CH_3), 1.15 (3H, d, $J = 7.9$ Hz, 21-CH_3), 1.00 (3H, s, 18-CH_3), 0.79 (3H, d, $J = 6.4$ Hz, 27-CH_3); ^{13}C-NMR spectral data was listed in Table 1; HRMS (FAB) m/z calc. for $C_{27}H_{40}O_3$ $[M + H]^+$, 413.3056. Found: 413.3057%.

*5.1.1.11. (20S,22S,25R)-Spirosol-1,4-diene-3-one (**20**).* A solution of **15** (500 mg, 1.0 mmol) and DDQ (90% purity) (1.0 g, 4.0 mmol) in absolute dioxane (20 mL) was refluxed for 36 h. The mixture was filtered, and the filtrate was evaporated. The resulting residue was dissolved in dichloromethane (100 mL), washed successively with 10% NaOH aqueous solution, water and brine, dried over anhydrous magnesium sulfate and then filtered. The organic filtrate was evaporated to obtain a residue that was subjected to a preparative HPLC (10% H_2O–acetone) to give 20 (224 mg, 49% yield) as needles, m.p. 200–203 °C (after recrystallization from methanol; lit [35], 199–202 °C): FABMA: m/z 433 $[M + Na]^+$. The ^{1}H-NMR spectral data was agreed with the data reported by Takahira et al. [35].

*5.1.1.12. (20S,22S,25R)-22-Thiospirosol-1,4-dien-3-one (**21**).* A solution of **17** (430 mg, 1.0 mmol) and DDQ (90% purity) (1.0 g, 4.0 mmol) in absolute dioxane (20 mL) was refluxed for 38 h. The mixture was filtered, and the filtrate was evaporated to give a residue that was dissolved in dichloromethane and filtered. The filtrate was washed successively with 10% NaOH aqueous solution, water and brine, dried over anhydrous magnesium sulfate and filtered. The filtrate was evaporated to give a residue that was subjected to preparative HPLC (10% H_2O–acetone) to obtain **21** (87 mg, 21% yield) as needles, m.p. 215–217 °C (after recrystallization from ethanol): FABMS: m/z 449 $[M + Na]^+$; ^{1}H-NMR ($CDCl_3$) (only assignable signals were

listed) δ 7.04 (1H, d, $J = 10.1$ Hz, H-1), 6.22 (1H, dd, $J = 10.1$, 1.8 Hz, H-2), 6.07 (1 H, s, H-4), 4.62 (1 H, dd, $J = 11.3$, 7.6 Hz, H-16), 2.53 (1H, dd, $J = 12.8$, 11.6 Hz, H-26a), 2.29 (1H, dd, $J = 12.8$, 2.4 Hz, H-26b), 1.25 (3H, s, 19-CH_3), 1.01 (3H, d, $J = 7.0$ Hz, 21-CH_3), 0.93 (3H, d, $J = 6.7$ Hz, 27-CH_3), 0.87 (3H, s, 18-CH_3); ^{13}C-NMR spectral data was listed in Table 1; HRMS (FAB) m/z calc. for $C_{27}H_{39}O_2S$ $[M + H]^+$, 427.2671. Found: 427.2664%.

*5.1.1.13. (22S,25R)-Spirosol-4-en-20-exomethylen-3-one (**22**).* The general procedure for the oxidation by DDQ was employed for **19** (315 mg, 0.76 mmol) to obtain a residue that was purified by a preparative HPLC (25% H_2O–acetone) to give **22** (93 mg: yield 23%) as needles, m.p. 212–214 °C (after recrystallization from ethanol): FABMS: m/z 433 $[M + Na]^+$; ^{1}H-NMR ($CDCl_3$) (only assignable signals were listed) δ 5.73 (1H, s, H-4), 5.18 (1H, d, $J = 2.1$ Hz, H-21 a), 5.01 (1H, d, $J = 2.1$ Hz, H-21b), 4.60 (1H, ddd, $J = 11.0$, 7.3, 4.6, Hz, H-16), 3.53 (1 H, ddd, $J = 11.0$, 4.9, 1.5 Hz, H-26a), 3.44 (1 H, t, $J = 11.0$ Hz, H-26b), 2.69 (1H, d, $J = 7.3$ Hz, H-17), 1.20 (3H, s, 19-CH_3), 0.82 (3H, d, $J = 6.4$ Hz, 27-CH_3), 0.74 (3H, s, 18-CH_3); ^{13}C-NMR spectral data was listed in Table 1; HRMS (FAB) m/z calc. for $C_{27}H_{39}O_3$ $[M + H]^+$, 411.2899. Found: 411.2900%.

*5.1.1.14. 26-Iodopseudodiosgenon (**23**).* The general procedure for Oppenauer oxidation was employed for **6** (1.0 g, 1.9 mmol) to obtain a residue that was subjected to a preparative HPLC (10% H_2O–acetone) to give **23** (725 mg, 73% yield) as yellowish semisolid: FABMS: m/z 545 $[M + Na]^+$; ^{1}H-NMR ($CDCl_3$) (only assignable signals were listed) δ 5.73 (1H, s, H-4), 4.74 (1H, ddd, $J = 10.1$, 7.9, 5.5 Hz, H-16), 3.25 (1H, dd, $J = 9.8$, 4.3 Hz, H-26a), 3.16 (1 H, dd, $J = 9.8$, 6.1 Hz, H-26b), 2.48 (1H, d, $J = 10.1$ Hz, H-17), 1.60 (3H, s, 21-CH_3), 1.20 (3H, s, 19-CH_3), 0.99 (3H, d, $J = 6.4$ Hz, 27-CH_3), 0.72 (3H, s, 18-CH_3); ^{13}C-NMR ($CDCl_3$) δ 199.4 (C-3), 171.0 (C-5), 151.3 (C-22), 123.9 (C-4), 103.9 (C-20), 84.1 (C-16), 64.0 (C-17), 54.2 (C-14), 53.7 (C-9), 43.3 (C-13), 39.3 (C-12), 38.6 (C-10), 35.7 (C-1), 35.0 (C-8), 34.1 (C-25), 33.9 (C-2), 33.7 (C-24), 32.8 (C-6), 32.3 (C-7 and C-15), 23.1 (C-11), 20.9 (C-23), 20.5 (C-27), 17.4 (C-19 and C-26), 14.1 (C-18), 11.6 (C-21); HRMS (FAB) m/z calc. for $C_{27}H_{40}IO_2$ $[M + H]^+$. 523.2073. Found: 523.2070%.

*5.1.1.15. 26-Thioacetylpseudodiosgenon (**24**).* The general procedure for thioacetylatioin was employed for **23** (300 mg, 0.57 mmol) to obtain a residue that was subjected to a preparative HPLC (15% H_2O–acetone) to give **24** (200 mg, 74% yield) as yellowish semisolid: FABMS: m/z 493 $[M + Na]^+$; ^{1}H-NMR ($CDCl_3$) (only assignable signals were listed) δ 5.73 (1H, s, H-4), 4.73 (1H, ddd, $J = 10.1$, 7.9, 5.5 Hz, H-16), 2.94 (1H, dd, $J = 13.4$, 5.5 Hz, H-26a), 2.78 (1H, dd, $J = 13.4$, 7.3 Hz, H-26b), 2.47 (1H, d, $J = 10.1$ Hz, H-17), 2.33 (3H, s, $SCOCH_3$), 1.59 (3H, s, 21-CH_3), 1.20 (3H, s, 19-CH_3), 0.95 (3H, d, $J = 6.7$ Hz, 27-CH_3), 0.71 (3H, s, 18-CH_3); ^{13}C-NMR ($CDCl_3$) δ 199.5 (C-3), 199.4 (S*C*OCH$_3$), 171.1 (C-5), 151.6 (C-22), 123.9 (C-4), 103.9 (C-20), 84.1 (C-16), 64.1 (C-17), 54.3 (C-14), 53.7 (C-9), 43.3 (C-13), 39.3 (C-12), 38.6 (C-10), 35.7 (C-1 and C-26), 35.0 (C-8), 34.0 (C-15), 33.9 (C-2), 33.3 (C-24), 32.8 (C-6 and C-25), 32.3 (C-7), 30.7 (SCO*C*H$_3$), 23.3 (C-11), 21.0 (C-23), 19.0 (C-27), 17.4 (C-19), 14.0 (C-18), 11.6 (C-21); HRMS (FAB) m/z calc. for $C_{29}H_{43}O_3S$ $[M + H]^+$, 471.2933. Found: 471.2931%.

*5.1.1.16. 26-Cyanoselenopseudodiosgenon (**25**).* The general procedure for selenocynation was employed for **23** (420 mg, 0.80 mmol) to give a residue that was subjected to a preparative HPLC (15% H_2O–acetone) to give **25** (270 mg, 67% yield) as yellowish semisolid: FABMS: m/z 524 $[M + Na]^+$; ^{1}H-NMR ($CDCl_3$) (only assignable signals were listed) δ 5.73 (1H, s, H-4), 4.75(1H, ddd, $J = 10.1$, 7.9, 5.8 Hz, H-16), 3.16 (1H, dd, $J = 11.9$, 5.5 Hz, H-26a), 2.96 (1H, dd, $J = 11.9$, 7.3 Hz, H-26b), 2.49 (1 H, d, $J = 10.1$ Hz, H-17), 1.60 (3H, s, 21-CH_3), 1.20 (3H, s, 19-CH_3), 1.08 (3H, d, $J = 6.7$ Hz, 27-CH_3), 0.72 (3H, s, 18-CH_3); ^{13}C-NMR ($CDCl_3$) δ 199.5 (C-3), 171.0 (C-5), 150.8 (C-22), 123.9 (C-4), 104.3 (C-20), 102.0 (SeCN), 84.2 (C-16), 64.0 (C-17), 54.2 (C-14), 53.7 (C-9), 43.3 (C-13), 39.3 (C-12), 38.6 (C-10), 35.6 (C-26), 35.7 (C-1), 35.0 (C-8), 33.9 (C-2 and C-5), 33.6 (C-25), 33.2 (C-24), 32.8 (C-6), 32.3 (C-7), 23.1 (C-11), 20.9 (C-23), 19.2 (C-27), 17.4 (C-19), 14.1 (C-18), 11.6 (C-21); HRMS (FAB) m/z calc. for $C_{28}H_{40}NO_2Se$ $[M + H]^+$, 502.2224. Found: 502.2218%.

5.1.2. Parmacology

5.1.2.1. Inhibitory activities of interferon-γ (INF-γ) production

Materials. Dimetylsulfoxide solution of steroidal compound was prepared at the concentration of 1 mM and stored at −20 °C before use. Rat anti-mouse CD-3 monoclonal antibody, hamster anti-mouse CD-28 monoclonal antibody and goat anti-rat IgG (γ) antibody were purchased from Serotec Ltd. (clone No KT 3) (England), PharMingen International (USA) and Kirkegaard and Perry Laboratories (Maryland, USA), respectively.

Assay procedure. $CD4^+$ cells were isolated by the MACS system [26] from BALB/c splenic T cells obtained according to the preparative method reported by Zhang et al. [27]. The $CD4^+$ cells were added with anti-CD3 (0.06 μg mL^{-1}) and anti-CD28 (1.0 μg mL^{-1}), and incubated at 37 °C under 5% CO_2 atmosphere for 3 days. The cell mixture was added with

mouse interleukin-2 (1.0 μg mL^{-1}), then further incubated at 37 °C under 5% CO_2 for 3 days. The proliferated cells were washed three times with Hanks solution, and suspended into RPMI 1640 medium containing 10% FCS at the density of 2×10^6 cells per mL. Each test compound (1, 3 or 10 μM), anti-CD3 (0.06 μg mL^{-1}) and anti-CD28 (1.0 μg mL^{-1}) were added in each 100 μL of the resulting suspension (ca. 10^6 cells), disseminated in a well of a 96-well flat-bottomed plate which was coated with anti-rat IgG (γ) antibody (10 μg mM^{-1}), and then incubated at 37 °C under 5% CO_2 for 24 h. After centrifugation (1200 rpm, 8 min, 4 °C, Hitachi, himac CR5DL), INF-γ activities were measured by a sand witch ELISA method [25].

5.1.2.2. Cytotoxic activities

Cells. Human colorectal (HCT 116) carcinoma cell line was purchased from ATCC (No. CCL-247). The cells were maintained in McCoy's 5A medium (12.0 g per H_2O (1000 mL) and $NaHCO_3$ (2.2 g) (Nissui, Tokyo, Japan) which were supplemented with 10% FBS at 37 °C in a humidified atmosphere containing 5% CO_2.

Assay procedure. Cells in the undergoing exponential growth were suspended in fresh medium at the concentration of 1×10^5 cells per mL (HCT 116) and inoculated in a 96-well flat-bottomed plate in a volume of 135 μL per well. Then, 15 μL aliquots of each sample were added to the wells. The cell survival was determined by the WST-1 assay. [28] Specifically, after the incubation at 37 °C for 72 h, a volume (25 μL) of the WST-1 solution (Cell counting kit; Wako pure chemichal Industries, Ltd., Japan) was immediately added to the cultured medium (135 μL) in each well. The cells were then incubated for another 4 h at 37 °C. The absorbance was measured at 450 nm by a microplate reader (Sjeia AUTO READER III, Sanko Junyaku, Co. Ltd., Japan). The experiment was repeated three times. IC_{50} values were calculated based on the analysis of the percentage inhibition of each sample at six or seven concentrations.

5.1.2.3. Antiurease activities

Materials. Phenol red solution (1 g L^{-1} phenol red in 47 vol% ethanol) was purchased from Kanto Chemical Co. Inc. (Tokyo, Japan). Urease (115 U mg^{-1} derived form Jack bean) was obtained from Wako Pure Chemical Industries Ltd. (Osaka, Japan). The control buffer of pH 7.7 was prepared by diluting a mixture of 1 M KH_2PO_4 (0.1 mL) and 1 M Na_2HPO_4 (0.7 mL) with H_2O to 80 mL. Also, the buffered solution of pH 6.7 was prepared by diluting a mixture of 1 M KH_2PO_4 (1 mL) and 1 M Na_2HPO_4 (1 mL) with H_2O to 100 mL. The buffered urea solution of pH 6.7 was obtained by dissolving urea (3 g) in a mixture of 1 M KH_2PO_4 (1 mL) and 1 M Na_2HPO_4 (1 mL) and then diluting the solution with H_2O to 100 mL.

Assay procedure. The antiurease activities were measured by modifying the colorimetric timing method by Van Slyke and Archibald [33] in the following way. Phenol red solution (50 μL) was added to blank solvent (H_2O, EtOH or DMSO; 100 μL) diluted with the control buffer of pH 7.7 (350 μL) in a 10×100 mm tube (referred to as **C** solution) and to sample solution (test compound in a blank slovent; 100 μL) diluted with the buffered solution of pH 6.7 (350 μL) in the same-type tube (referred to as **S** solution), respectively. The resultant **C** and **S** solutions, after the addition of the urrease solution (575 units per 5 mg in H_2O (24.8 mL); 500 μL), were pre-incubated at 30 °C for 30 min, respectively. The **C** solution was diluted with the control buffer of pH 7.7 (5 mL), whose absorbance at 560 nm measured by a spectrophotometer was adjusted to 0. The **S** solution was diluted with the buffered urea solution of pH 6.7 (5 mL). A time interval for the absorbance of the **C** solution to reach 0 was measured at 560 nm by using a stop-watch. The percentage inhibition (%) of each sample was calculated according to the following equation:

$$\text{percentage inhibition } (\%) = [(t - t_0)/t] \times 100$$

t, time interval (s) measured at each molar sample concentration; t_0, time interval (s) measured at zero molar sample concentration.

5.1.2.4. Antibacterial activities

Materials. Steroidal compounds **3**, **4**, **8**, **9**, **11**–**18**, **20**, **21**, **24** and **25** were dissolved in DMSO at the concentration of 25 μg mL^{-1} and stored at 4 °C before use. A tumbling-defective mutant of *B. subtilis* 168 UV [29] was used for the bacterial motion analysis and its wild-type 168 *trp* and *E. coli* RP 470, the latter of which was kindly supplied by Adler [32], were employed for the disk assay. Cells were cultivated in L broth (pH 7.0) to the middle logarithmic growth phase, as described above [29,31].

Assay procedure. Antimicrobial efficacy was determined by either a bacterial motion speed analysis or a disk assay. In the former, solution of compound was added to 2 mL of the bacterial culture at a final concentration of 25 μg mL^{-1}. Then, the specimen for microscopic ovservation was prepared as described above [29,31,34]. The motion speed of cells was measured by using a motion analysis system comprising of a microscope (BH2, Olympus), a CCD camera (Tl-23A, NEC Co.), an image analyzer (MD-20R, Keio Electronic Ind) and a personal computer with a MOTION ANALYSIS PROGRAM, (MAS), version 1.22 (Keio Electronic Ind.). The bright area of a bacterial cell image on a dark background was processed by the analyzer and recorded on a grid of 241 vertical by 256 horizontal pixels (6.25 pixels μm^{-1}). The total of 8–15 scenes, each containing one to five cells, was processed for an image analysis.

For the disk assay, the culture was suspended in motion L agar (0.7%), and 5 mL of its suspension was overlaid on 5 mL of L agar (1.5%) solidified in petri dish. Then, a paper disk, which was immersed into a solution (40 mL) of each compound at a concentration of 25 μg mL^{-1} and dried, was placed on the above agar plate. After the plate was incubated at 37 °C for 20 h, the diameter of inhibition zone fomed was measured.

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