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Ten More Ritterazines, Cytotoxic Steroidal Alkaloids from the Tunicate *Ritterella tokioka*[1]

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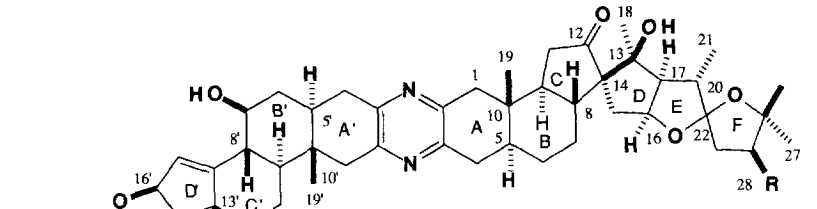
Abstract: Ritterazines D-M (**4**-**13**) have been isolated from the tunicate *Ritterella tokioka* and their structures elucidated by spectral data. Ritterazines D-M showed potent cytotoxicity against P388 murine leukemia cells with IC_{50} values of 16, 3.5, 0.73, 0.73, 16, 14, 13, 9.5, 10, and 15 ng/mL, respectively.

Marine natural products are potential anticancer agents, some of which are now under preclinical studies.[2] Although marine sponges have been most frequently studied,[3] tunicates also proved to be an important source of antitumor/cytotoxic metabolites[4] as examplified by the didemnins,[5, 6] the ecteinascidins,[7, 8] and the patellazoles.[9, 10] In our continuing search for cytotoxic substances from Japanese marine invertebrates,[1] we found potent activity in the lipophilic extract of the tunicate *Ritterella tokioka* (family Polyclinidae) collected off the Izu Peninsula, from which we isolated ritterazines A (**1**), B (**2**), and C (**3**),[11, 12] dimeric steroidal alkaloids closely related to cephalostatins reported from the East African hemichordate *Cephalodiscus gilchristi*.[13-18] Further fractionation of the extract of *R. tokioka* afforded 10 more related compounds, ritterazines D-M (**4**-**13**), which is the subject of this paper.

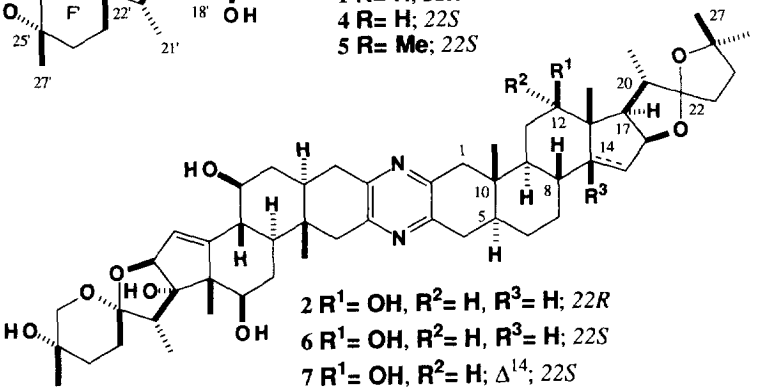
Colonies of the tunicate[19] (8.2 kg) were extracted with EtOH. The combined extracts were concentrated and partitioned between water and ethyl acetate The organic phase was fractionated by the Kupchan partitioning procedure;[20] most of the cytotoxicity against P388 murine leukemia cells was found in the CH_2Cl_2 phase. The CH_2Cl_2 soluble material was repeatedly purified by ODS and Sephadex LH-20 chromatographies to yield ritterazines D (**4**), E (**5**), F (**6**), G (**7**), H (**8**), I (**9**), J (**10**), K (**11**), L (**12**), and M (**13**) (yields, 4.0, 2.8, 2.6, 2.2, 1.2, 4.4, 2.8, 6.2, 1.1, and 1.9 mg, respectively) as colorless glassy solids.

Ritterazine D (**4**) had the same molecular formula of $C_{54}H_{76}N_2O_{10}$ as ritterazine A as determined by HR-FABMS. The UV spectrum[21] [λ_{max} 286 nm (ε 9200)] suggested the presence of a pyrazine ring. This was substantiated by ^{13}C NMR signals at δ 148.6, 148.9, 148.9, and 149.0.[22] The gross structure of ritterazine D obtained by interpretation of DQF-COSY, HMQC,[23] and HMBC[24] data was identical with that of ritterazine A.[11] Significant differences in ^1H and ^{13}C NMR chemical shift values between the two compounds were observed for signals around C20 in the eastern hemisphere; NMR data for the western hemisphere were superimposable on each other.

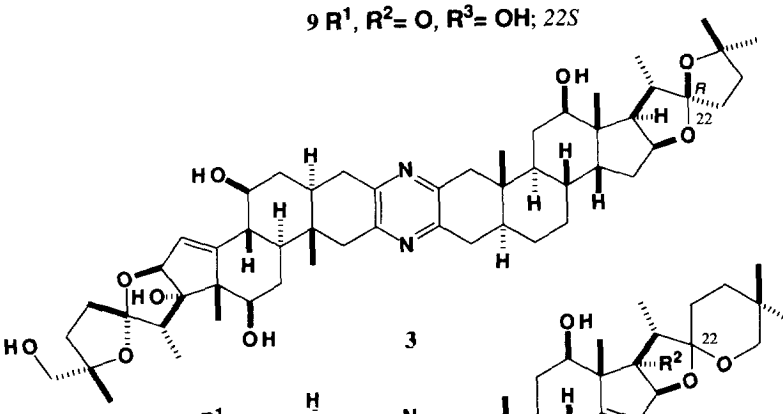
The relative stereochemistry of **4** was determined by NOESY data. The NOESY spectrum of **4** exhibited the same sets of cross peaks that were observed for the western hemisphere of **1**. Therefore, the western hemisphere of **4** had the relative stereochemistry identical with that of **1**. With respect to the relative stereochemistry of the eastern hemisphere, *trans*-fusion of rings A and B was deduced on the basis of ^{13}C chemical shift of C19 at 11.1 ppm.[26] NOESY cross peaks: 19-CH_3/H8, 13-OH/H8, and H7α/H15β indicated



1 R= H; *22R*
4 R= H; *22S*
5 R= **Me**; *22S*



2 R^1= OH, R^2= H, R^3= H; *22R*
6 R^1= OH, R^2= H, R^3= H; *22S*
7 R^1= OH, R^2= H; Δ^{14}; *22S*
8 R^1, R^2= O, R^3= H; *22R*
9 R^1, R^2= O, R^3= OH; *22S*



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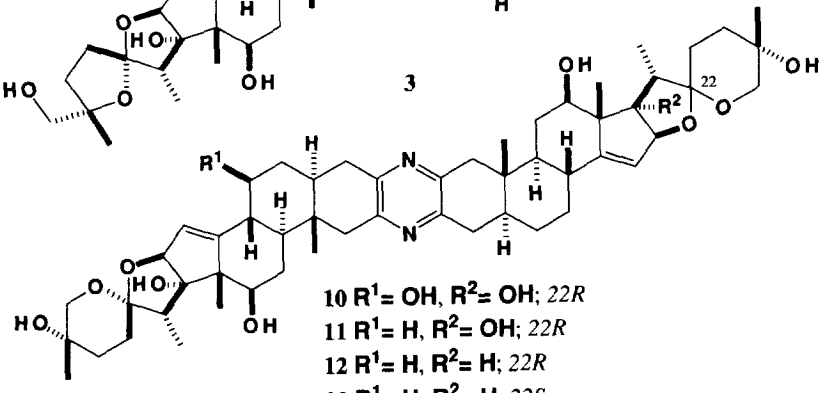

10 R^1= OH, R^2= OH; *22R*
11 R^1= H, R^2= OH; *22R*
12 R^1= H, R^2= H; *22R*
13 R^1= H, R^2= H; *22S*

14*R* stereochemistry, while NOESY cross peaks: 13-OH/H20 and H16/H17 demonstrated the same relative stereochemistry in rings D and E as that of ritterazine A.

In our previous paper, stereochemistry at C22 in ritterazine A was arbitrarily chosen, because no cross peak was observed between protons in rings E and F in the NOESY spectrum measured at 300 K in pyridine-d_5. When we realized that ritterazines A and D were isomeric at C22, we attempted to obtain better resolved NOESY spectra of **1** and **4** under different measuring conditions, among which lowering of the temperature to 263 K in pyridine-d_5 was most satisfactory. At this temperature, ritterazine A (**1**) gave a cross peak between 21-CH_3 and H23β suggesting 22*R* stereochemistry, whereas a cross peak between H20 and H23β in **4** was in agreement with 22*S* stereochemistry. (Figure 1)

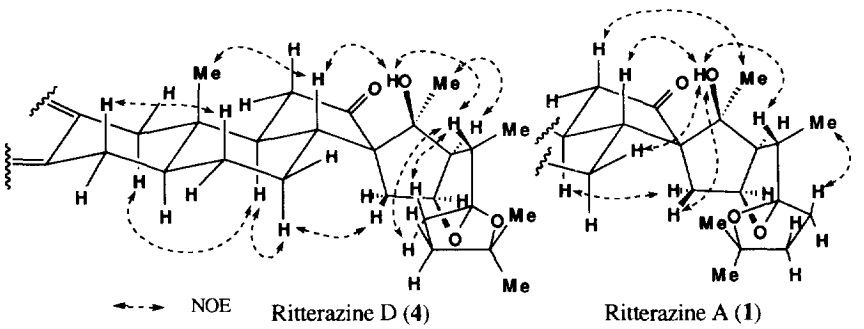

Figure 1. Comparison of NOESY data of **1** and **4.**

Ritterazine E (**5**) had a molecular formula one CH_2 unit larger than **4**. The ^1H NMR spectrum of **5** displayed one additional doublet methyl signal; the other signals were almost superimposable. Interpretation of 2D NMR data showed that ritterazine E was 24-methylritterazine D. The NOESY spectrum exhibited cross peaks: 28-CH_3/H23β and H23β/H20, which allowed assignment of 24*S* stereochemistry. The rest of the molecule was identical with **4**.

Ritterazine F (**6**) showed an $(M+H)^+$ ion at *m/z* 899.5764 in HR-FABMS, matching a molecular formula of $C_{54}H_{79}N_2O_9$ (Δ -2.1 mmu). The gross structure of ritterazine F was the same as that of ritterazine B (**2**). NMR data of the two compounds were different around C22 [**6**/**2**; δ_C 40.7/42.1 (C20); 16.3/14.7 (C21); 30.1/33.2 (C23): δ_H 2.84/3.15 (H17); 2.29/2.01 (H20); 1.08/1.18 (H21), 1.87/2.12 (H23β)]. The NOESY spectrum of **6** exhibited the same sets of cross peaks that were observed for **2** except for cross peaks between protons in rings E and F. The stereochemistry of C22 was assigned on the basis of ROESY[27, 28] data , which gave a cross peak between H20 and H23β, suggesting 22*S* stereochemistry.[29]

Ritterazine G (**7**) had one more unsaturation than **6**, as observed in HR-FABMS. The DQF-COSY, HMQC, HMBC, and NOESY spectra of **7** exhibited the same sets of cross peaks that were observed for the western hemispheres of **2** and **6**. Except for the presence of the Δ^{14} olefin, the NOESY spectrum of **7** exhibited the same sets of cross peaks that were observed for ritterazine F (**6**). Assignment of the stereochemistry at C22 was made by measuring the ROESY spectrum in pyridine-d_5 at 300 K, which gave a cross peak between H20 and H23β, suggesting 22*S* stereochemistry.

Table I. ^{13}C NMR Data of Ritterazines D-M (pyridine-d_5)

No.	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**
1	46.6 t	46.5 t	46.1 t	46.2 t	45.8 t	45.6 t	46.0 t	46.0 t	45.8 t	45.9 t
2	148.9 s	149.0 s	149.0 s	149.0 s	148.8 s	148.9 s	148.8 s	148.6 s	148.9 s	148.9 s
3	149.0 s	148.9 s	149.0 s	148.3 s	148.8 s	148.9 s	148.8 s	148.7 s	149.0 s	148.9 s
4	35.4 t	35.5 t	35.6 t	35.8 t	36.0 t	35.5 t	35.7 t	35.8 t	35.5 t	35.6 t
5	41.6 d	41.7 d	41.4 d	41.8 d	41.1 d	41.3 d	41.8 d	41.7 d	41.5 d	41.5 d
6	28.6 t	28.6 t	28.7 t	28.4 t	29.0 t	28.1 t	28.3 t	28.2 t	28.0 t	28.1 t
7	30.5 t	32.5 t	31.4 t	29.7 t	31.4 t	27.2 t	29.0 t	28.9 t	29.4 t	29.8 t
8	40.8 d	40.7 d	32.4 d	33.8 d	48.1 d	40.5 d	34.0 d	34.0 t	33.7 d	34.5 d
9	50.3 d	50.0 d	45.6 d	52.7 d	47.9 d	46.7 d	53.0 d	52.9 d	52.6 d	49.5 d
10	35.7 s	35.6 s	35.9 s	36.2 s	35.8 s	36.5 s	36.1 s	36.3 s	36.5 s	36.8 s
11	40.8 t	40.8 t	30.6 t	30.9 t	37.7 t	37.2 t	29.2 t	29.2 t	30.7 t	29.3 t
12	220.1 s	220.7 s	72.0 d	78.9 d	214.5 s	213.5 s	75.8 d	75.6 d	78.9 d	76.3 d
13	80.0 s	80.0 s	49.0 s	53.5 s	57.8 s	62.5 s	56.0 s	56.0 s	53.7 s	52.9 s
14	70.5 s	70.8 s	47.3 d	152.0 s	49.2 d	87.0 s	155.0 s	154.8 s	157.9 s	154.0 s
15	33.9 t	33.8 t	33.2 t	120.9 d	32.5 t	38.6 t	120.2 s	120.0 d	120.0 d	119.0 d
16	80.0 d	80.2 d	78.4 d	85.0 d	77.7 d	79.2 d	93.9 d	93.7 d	85.4 d	86.9 d
17	60.5 d	60.5 d	56.9 d	56.2 d	52.2 d	53.1 d	93.3 s	93.3 s	56.6 d	54.4 d
18	23.7 q	23.7 q	14.0 q	13.8 q	19.4 q	15.1 q	12.7 q	13.0 q	13.8 q	18.8 q
19	11.1 q	10.8 q	11.7 q	11.8 q	11.9 q	11.3 q	11.6 q	11.7 q	11.7 q	11.5 q
20	38.4 d	37.7 d	40.7 d	42.1 d	41.1 d	42.1 d	48.2 d	48.4 d	44.9 d	45.0 d
21	14.7 q	14.7 q	16.3 q	14.4 q	17.0 q	15.0 q	8.0 q	8.2 q	14.2 q	14.5 q
22	120.7 s	118.6 s	117.8 s	117.9 s	117.5 s	117.8 s	108.0 s	107.9 s	107.3 s	107.1 s
23	32.8 t	41.0 t	30.1 t	33.8 t	33.1 t	32.9 t	27.4 t	27.7 t	27.5 t	27.5 t
24	37.9 t	41.6 d	37.0 t	37.8 t	37.4 t	37.3 t	33.3 t	33.3 t	33.1 t	33.4 t
25	81.4 s	83.5 s	81.0 s	82.6 s	81.2 s	81.7 s	65.8 s	65.7 s	66.2 s	66.0 s
26	30.3 q	23.3 q	29.7 q	28.7 q	28.9 q	28.3 q	70.4 t	70.2 t	70.0 t	70.2 t
27	28.5 q	29.6 q	28.4 q	30.3 q	30.3 q	30.0 q	26.9 q	27.0 q	26.8 q	26.8 q
28		14.0 q								
1'	46.0 t	46.8 t	45.9 t	46.0 t	46.0 t	45.6 t	46.0 t		45.8 t	45.8 t
2'	148.6 s	148.9 s	148.9 s	148.3 s	148.5 s	148.6 s	148.8 s		148.9 s	148.9 s
3'	148.9 s	149.0 s	148.9 s	149.0 s	148.5 s	148.6 s	148.8 s		149.0 s	148.9 s
4'	35.4 t	35.5 t	35.6 t	35.7 t	36.0 t	35.3 t	35.7 t		35.5 t	35.6 t
5'	40.0 d	40.0 d	39.8 d	40.0 d	40.2 d	39.7 d	40.0 d		41.5 d	41.5 d
6'	38.3 t	38.7 t	38.2 t	38.5 t	38.6 t	38.1 t	38.4 t		28.0 t	28.1 t
7'	69.3 d	69.6 d	69.0 d	69.6 d	69.5 d	69.3 d	69.5 d		28.8 t	28.7 t
8'	42.7 d	43.8 d	42.6 d	42.9 d	43.1 d	42.5 d	42.8 d		33.8 d	33.7 d
9'	51.3 d	51.1 d	51.1 d	51.3 d	51.2 d	51.1 d	51.1 d		52.6 d	52.6 d
10'	36.0 s	35.8 s	35.9 s	35.9 s	36.1 s	35.9 s	35.9 s		36.5 s	36.3 s
11'	29.0 t	29.0 t	29.0 t	29.3 t	29.4 t	28.9 t	29.2 t		29.0 t	28.9 t
12'	75.5 d	75.8 d	75.8 d	75.8 d	75.6 d	75.9 d	75.8 d		75.8 d	75.8 d
13'	56.2 s	56.0 s	56.0 s	56.0 s	56.0 s	56.1 s	56.0 s		56.8 s	56.1 s
14'	151.7 s	151.3 s	151.6 s	151.6 s	151.6 s	151.6 s	151.8 s		155.0 s	155.0 s
15'	121.0 d	120.2 d	121.4 d	121.7 d	120.9 d	121.0 d	121.3 d		119.0 d	120.7 d
16'	93.6 d	94.0 d	94.1 d	93.8 d	93.9 d	94.0 d	94.2 d		94.0 d	93.7 d
17'	93.5 s	93.2 s	93.2 s	93.2 s	93.1 s	93.3 s	93.3 s		93.3 s	93.1 s
18'	12.6 q	12.4 q	12.3 q	12.5 q	12.9 q	12.2 q	12.7 q		12.8 q	12.5 q
19'	11.8 q	11.7 q	11.6 q	11.8 q	12.2 q	11.5 q	11.6 q		11.7 q	11.5 q
20'	48.1 d	48.1 d	48.0 d	48.1 d	48.4 d	47.9 d	48.2 d		48.2 d	48.2 d
21'	8.2 q	7.8 q	7.9 q	8.0 q	8.4 q	7.8 q	8.0 q		7.8 q	7.9 q
22'	108.0 s	108.0 s	108.0 s	108.0 s	108.0 s	108.0 s	108.0 s		108.2 s	108.0 s
23'	27.3 t	27.4 t	27.3 t	27.6 t	27.7 t	27.1 t	27.4 t		27.5 t	27.5 t
24'	33.5 t	33.2 t	33.2 t	33.5 t	33.5 t	33.1 t	33.3 t		33.5 t	33.1 t
25'	65.6 s	65.6 s	65.8 s	65.9 s	65.7 s	65.7 s	65.8 s		66.0 s	66.5 s
26'	70.0 t	70.2 t	70.2 t	70.2 t	70.2 t	70.2 t	70.4 t		70.0 t	70.2 t
27'	26.8 q	26.7 q	26.8 q	27.0 q	27.2 q	26.6 q	26.9 q		26.8 q	26.8 q

A ketone signal (δ 214.5) was exhibited in the ^{13}C NMR spectrum of ritterazine H (**8**). NMR data of the western hemisphere of **8** were identical with those of **2**. The ketone was placed at C12, which was evident from HMBC cross peaks between C12 and H11α, H11β, H14, H17, and H18. The NOESY spectrum revealed that the relative stereochemistry of **8** was identical with that of **2**.

Table II. ^{1}H NMR Data of Ritterazines D-M (eastern hemisphere)

No.	4	5	6	7	8	9	10	11	12	13
1α	2.72 d[a]	2.72 d[e]	2.72 d[l]	2.64 d[r]	2.60 d[a']	2.67 d[l']	2.66 d[x']	2.67 d[h'']	2.67 d[r'']	2.64 d[c''']
1β	2.87 d[b]	2.87 d[f]	3.17 d[m]	3.10 d[s]	2.97 d[b']	3.05 d[m']	3.13 d[y']	3.13 d[i'']	3.13 d[s'']	3.10 d[d''']
4α	2.90 m	2.90 ddg	2.95 dd[n]	2.92 dd[t]	2.89 dd[c']	2.94 dd[n']	2.93 dd[z']	2.92 dd[J'']	2.91 dd[t'']	2.91 dd[e''']
4β	2.57 m	2.57 dd[h]	2.68 dd[o]	2.67 dd[u]	2.61 dd[d']	2.66 dd[o']	2.67 dd[a'']	2.67 m	2.66 m	2.65 m
5	1.64 m	1.64 m	1.56 m	1.61 m	1.48 m	1.64 m	1.63 m	1.63 m	1.64 m	1.64 m
6α	1.47 m	1.48 m	1.46 m	1.54 m	1.42 m	1.56 m	1.54 m	1.55 m	1.55 m	1.55 m
6b	1.16 m	1.15 m	1.21 m	1.27 m	1.20 m	1.26 m	1.27 m	1.27 m	1.28 m	1.28 m
7α	1.32 m	1.37 m	1.05 m	1.33 m	1.00 m	1.17 m	1.36 m	1.35 m	1.38 m	1.55 m
7β	2.34 m	2.34 m	1.40 m	1.81 m	1.40 m	2.42 m	1.75 m	1.75 m	1.85 m	1.86 m
8	2.35 m	2.35 m	1.61 m	2.16 m	1.95 m	2.09 m	2.09 m	2.09 m	2.14 m	2.23 m
9	1.60 m	1.32 m	1.34 m	0.92 m	1.62 m	1.43 m	0.98 m	0.99 ddd[k'']	1.00 m	1.77 m
11α	2.37 dd[c]	2.37 m	2.07 m	2.10 m	2.45 dd[e']	2.54 dd[p']	2.15 m	2.13 m	2.13 m	1.91 m
11β	2.15 m	2.15 m	1.67 m	1.88 m	2.55 dd[f']	2.73 dd[q']	1.81 m	1.82 m	1.90 m	1.77 m
12			3.60 m	3.48 dd[v]			4.19 dd[b'']	4.19 dd[l'']	3.52 dd[u'']	4.03 m
14			2.28 m		2.52 d[g']					
15α	2.09 m	2.10 m	1.84 m	5.55 s	1.24 m	1.67 dd[r']	5.60 s	5.60 s	5.63 brs	5.61 brs
15β	2.34 m	2.37 m	2.01 m		1.73 m	2.05 d[s']				
16	5.27 m	5.30 ddd[i]	4.48 ddp	5.27 brd[w]	4.31 dd[h']	4.79 dd[t']	5.18 s	5.18 s	5.25 dd[v'']	5.46 brd[f''']
17	2.98 m	2.98 m	2.84 m	3.09 q[x]	3.22 dd[i']	3.74 dd[u']			3.18 dd[w'']	3.32 dd[g''']
18	1.44 s	1.45 s	1.26 s	1.32 s	1.21 s	1.50 s	1.33 s	1.33 s	1.32 s	1.23 s
19	0.73 s	0.73 s	0.69 s	0.76 s	0.70 s	0.76 s	0.77 s	0.78 s	0.77 s	0.80 s
20	3.01 m	3.00 m	2.29 m	2.22 dq[y]	2.08 dq[j']	2.90 dq[v']	2.22 qc''	2.21 q[m'']	2.11 dq[x'']	2.08 m
21	1.14 d[d]	1.14 d[j]	1.08 d[q]	1.25 d[z]	0.94 d[k']	1.09 d[w']	1.29 d[d'']	1.29 d[n'']	1.38 d[y'']	1.23 d[h''']
23α	1.64 m	1.74 m	1.75 m	1.68 m	1.75 m	1.62 m	2.56 ddd[e'']	2.50 ddd[o'']	2.57 ddd[z'']	2.56 ddd[i''']
23β	2.02 m	2.15 m	1.87 m	2.12 m	1.85 m	1.99 m	1.59 m	1.58 m	1.64 m	1.64 m
24α	1.62 m	2.44 m	1.67 m	1.67 m	1.67 m	1.88 m	1.87 m	1.88 m	1.86 m	1.90 m
24β	2.00 m		2.05 m	2.03 m	1.97 m	2.17 m	2.17 m	2.18 m	2.18 m	2.20 m
26α	1.42 s	1.02 s	1.20 s	1.19 s	1.14 s	1.17 s	3.62 brd[f'']	3.62 brd[p'']	3.75 dd[a''']	3.72 dd[J''']
26β							4.01 dg''	4.02 dq''	4.08 d[b''']	4.05 d[k''']
27	1.20 s	1.39 s	1.44 s	1.46 s	1.36 s	1.41 s	1.23 s	1.22 s	1.26 s	1.24 s
28		0.82 d[k]								
12OH							4.72 brs	4.72 s		
13OH	6.01 s	6.00 s								
17OH							5.10 s	5.08 s		
25OH										

a. *J*= 16.0 Hz, b. *J*= 16.0 Hz, c. *J*= 16.5, 6.3 Hz, d. *J*= 6.5 Hz, e. *J*= 16.5 Hz, f. *J*= 16.5 Hz, g. *J*= 17.0, 5.0 Hz, h. *J*= 17.0, 12.5 Hz, i. *J*= 9.3, 3.9, 2.9 Hz, j. *J*= 6.0 Hz, k. *J*= 7.0 Hz, l. *J*= 17.0 Hz, m. *J*= 17.0 Hz, n. *J*= 15.0, 5.5 Hz, o. *J*= 15.0, 12.5 Hz, p. *J*= 7.0, 6.5 Hz, q. *J*= 7.0 Hz, r. *J*= 17.5 Hz, s. *J*= 17.5 Hz, t. *J*= 18.0, 5.0 Hz, u. *J*= 18.0, 11.0 Hz, v. *J*= 11.5, 4.5 Hz, w. *J*= 8.0 Hz, x. *J*= 8.0 Hz, y. *J*= 8.0, 7.0 Hz, z. *J*= 7.0 Hz, a'. *J*= 16.0 Hz, b'. *J*= 16.0 Hz, c'. *J*= 17.5, 5.5 Hz, d'. *J*= 17.5, 11.0 Hz, e'. *J*= 13.0, 4.5 Hz, f'. *J*= 13.0, 12.0 Hz, g'. *J*= 13.5 Hz, h'. *J*= 7.0, 7.0 Hz, i'. *J*= 7.5, 7.0 Hz, j'. *J*= 7.5, 7.0 Hz, k'. *J*= 7.0 Hz, l'. *J*= 16.5 Hz, m'. *J*= 16.5 Hz, n'. *J*= 15.0, 5.6 Hz, o'. *J*= 15.0, 11.4 Hz, p'. *J*= 13.5, 4.0 Hz, q'. *J*= 13.5, 13.5 Hz, r'. *J*= 15.0, 7.5 Hz, s'. *J*= 15.0 Hz, t'. *J*= 8.0, 7.5 Hz, u'. *J*= 9.0, 8.0 Hz, v'. *J*= 9.0, 7.0 Hz, w'. *J*= 7.0 Hz, x'. *J*= 17.0 Hz, y'. *J*= 17.0 Hz, z'. *J*= 17.5, 5.0 Hz, a''. *J*= 17.5, 12.0 Hz, b''. *J*= 11.5, 4.5 Hz, c''. *J*= 6.5 Hz, d''. *J*= 6.5 Hz, e''. *J*= 14.0, 13.0, 4.5 Hz, f''. *J*= 11.5 Hz, g''. *J*= 11.5 Hz, h''. *J*= 16.5 Hz, i''. *J*= 16.5 Hz, j''. *J*= 17.5, 5.5 Hz, k''. *J*= 12.5, 11.5, 4.0 Hz, l''. *J*= 11.2, 4.6 Hz, m''. *J*= 6.5 Hz, n''. *J*= 6.5 Hz, o''. *J*= 13.5, 13.0, 4.5 Hz, p''. *J*= 11.0 Hz, q''. *J*= 11.0 Hz, r''. *J*= 16.5 Hz, s''. *J*= 16.5 Hz, t''. *J*= 17.5, 5.5 Hz, u''. *J*= 11.0, 4.5 Hz, v''. *J*= 8.0, 1.2 Hz, w''. *J*= 8.5, 8.0 Hz, x''. *J*= 8.5, 7.5 Hz, y''. *J*= 7.5 Hz, z''. *J*= 13.0, 13.0, 5.0 Hz, a'''. *J*= 11.0, 2.0 Hz, b'''. *J*= 11.0 Hz, c'''. *J*= 17.0 Hz, d'''. *J*= 17.0 Hz, e'''. *J*= 12.0, 5.0 Hz, f'''. *J*= 8.0 Hz, g'''. *J*= 8.5, 8.0 Hz, h'''. *J*= 7.0 Hz, i'''. *J*= 7.0 Hz, j'''. *J*= 11.5, 2.0 Hz, k'''. *J*= 11.5 Hz,

Ritterazine I (**9**) had one more oxygen atom than **8**. The gross structure of ritterazine I was identical with that of **8** except for the presence of 14-OH, which was implied by the ^{13}C NMR chemical shift of C14 at 87.0 ppm. The NOESY spectrum of **9** exhibited the same sets of cross peaks as those observed for **8**, except for protons on rings E and F. A cross peak between H20 and H23β in the ROESY spectrum suggested 22*S* stereochemistry.

Table III. ^{1}H NMR Data of Ritterazines D-M (western hemisphere)

No.	4	5	6	7	8	9	10	12	13
1'α	2.68 d[a]	2.68 d[h]	2.68 d[s]	2.68 d[d']	2.63 d[p']	2.68 d[a'']	2.69 d[m'']	2.67 d[x'']	2.66 dg['''']
1'β	3.15 d[b]	3.15 d[i]	3.15 d[t]	3.15 d[e']	3.11 d[q']	3.15 d[b'']	3.16 d[n'']	3.13 d[y'']	3.12 d[h'''']
4'α	3.01 m	3.00 dd[j]	2.98 dd[u]	2.98 dd[f']	2.95 dd[r']	2.99 dd[c'']	3.00 dd[o'']	2.94 dd[z'']	2.88 dd[i'''']
4'β	2.74 m	2.79 dd[k]	2.77 dd[v]	2.77 dd[g']	2.74 dd[s']	2.77 dd[d'']	2.78 dd[p'']	2.66 m	2.66 m
5'	1.87 m	1.88 m	1.87 m	1.87 m	1.83 m	1.88 m	1.88 m	1.64 m	1.64 m
6'α	2.21 m	2.21 m	2.20 m	2.20 m	2.16 m	2.21 m	2.21 m	1.55 m	1.55 m
6'β	1.76 m	1.76 m	1.75 m	1.76 m	1.70 m	1.75 m	1.76 m	1.28 m	1.28 m
7'α	4.06 m	4.07 m	4.06 m	4.06 ddd[h']	4.02 m	4.06 ddd[e'']	4.06 m	1.37 m	1.36 m
7'β								1.75 m	1.76 m
8'	2.42 m	2.42 dd[l]	2.41 dd[w]	2.42 dd[i']	2.37 dd[t']	2.42 dd[f'']	2.42 dd[q'']	2.09 m	2.09 m
9'	1.18 m	1.17 m	1.17 m	1.17 m	1.14 m	1.09 m	1.17 m	0.99 m	0.97 m
11'α	2.16 m	2.18 m	2.18 m	2.12 m	2.13 m	2.09 m	2.17 m	2.13 m	2.13 m
11'β	1.87 m	1.88 m	1.87 m	1.87 m	1.83 m	1.88 m	1.88 m	1.82 m	1.87 m
12'	4.21 m	4.21 dd[m]	4.21 dd[x]	4.21 dd[j']	4.17 dd[u']	4.21 dg['']	4.21 dd[r'']	4.19 dd[a''']	4.19 dd[j''']
15'	6.14 s	6.25 s	6.18 s	6.13 s	6.09 s	6.14 s	6.14 s	5.61 s	5.85 s
16'	5.26 s	5.26 s	5.26 s	5.26 s	5.22 s	5.26 s	5.26 s	5.19 s	5.19 s
18'	1.33 s	1.33 s	1.33 s	1.33 s	1.29 s	1.33 s	1.34 s	1.33 s	1.33 s
19'	0.86 s	0.86 s	0.84 s	0.84 s	0.80 s	0.84 s	0.85 s	0.78 s	0.77 s
20'	2.22 q[c]	2.22 q[n]	2.21 q[y]	2.22 q[k']	2.17 q[v']	2.22 q[h'']	2.22 qs''	2.21 q[b''']	2.21 q[k''']
21'	1.26 d[d]	1.26 d[o]	1.26 d[z]	1.26 d[l']	1.22 d[w']	1.26 d[i'']	1.27 d[t'']	1.29 d[c''']	1.29 d[l''']
23'α	2.51 ddd[e]	2.51 ddd[p]	2.51 ddd[a']	2.51 ddd[m']	2.46 ddd[x']	2.50 ddd[j'']	2.51 ddd[u'']	2.56 ddd[d''']	2.56 ddd[m''']
23'β	1.45 m	1.45 m	1.46 m	1.46 m	1.40 m	1.45 m	1.45 m	1.59 m	1.57 m
24'α	1.87 m	1.88 m	1.87 m	1.87 m	1.41 m	1.98 m	1.86 m	1.90 m	1.87 m
24'β	2.16 m	2.16 m	2.15 m	2.12 m	2.12 m	2.16 m	2.16 m	2.19 m	2.18 m
26'	3.61 brd[f]	3.61 dd[q]	3.61 dd[b']	3.61 dd[n']	3.56 dd[y']	3.61 dd[k'']	3.62 brd[v'']	3.62 dd[e''']	3.62 dd[n''']
	4.01 dg	4.01 d[r]	4.01 d[c']	4.01 d[o']	3.96 d[z']	4.01 d[l'']	4.01 d[w'']	4.02 d[f''']	4.02 d[o''']
27'	1.22 s	1.22 s	1.22 s	1.22 s	1.18 s	1.22 s	1.23 s	1.23 s	1.23 s
12'OH	4.69 s						4.70 brs		
17'OH	5.06 s		5.06 s	5.08 s	5.03 s	5.07 s	5.06 s	5.11 s	5.11 s
25'OH	3.62 s								

a. *J*= 15.0 Hz, b. *J*= 15.0 Hz, c. *J*= 7.0 Hz, d. *J*= 7.0 Hz, e. *J*= 13.3, 13.1, 4.9 Hz, f. *J*= 11.0 Hz, g. *J*= 11.0 Hz, h. *J*= 16.5 Hz, i. *J*= 16.5 Hz, j. *J*= 16.0, 5.0 Hz, k. *J*= 16.0, 12.5 Hz, l. *J*= 11.5, 10.5 Hz, m. *J*= 11.0, 4.0 Hz, n. *J*= 7.0 Hz, o. *J*= 7.0 Hz, p. *J*= 14.0, 13.5, 5.0 Hz, q. *J*= 12.0, 2.0 Hz, r. *J*= 12.0 Hz, s. *J*= 16.5 Hz, t. *J*= 16.5 Hz, u. *J*= 17.5, 5.5 Hz, v. *J*= 17.5, 10.5 Hz, w. *J*= 10.0, 9.0 Hz, x. *J*= 10.5, 4.0 Hz, y. *J*= 6.5 Hz, z. *J*= 6.5 Hz, a'. *J*= 14.0, 13.0, 4.5 Hz, b'. *J*= 11.5, 2.0 Hz, c'. *J*= 11.5 Hz, d'. *J*= 17.0 Hz, e'. *J*= 17.0 Hz, f'. *J*= 17.5, 5.5 Hz, g'. *J*= 17.5, 11.5 Hz, h'. *J*= 10.0, 10.0, 4.0 Hz, i'. *J*= 11.0, 10.0 Hz, j'. *J*= 11.0, 4.5 Hz, k'. *J*= 7.0 Hz, l'. *J*= 7.0 Hz, m'. *J*= 13.5, 13.0, 4.0 Hz, n'. *J*= 11.5, 2.0 Hz, o'. *J*= 11.5 Hz, p'. *J*= 16.5 Hz, q'. *J*= 16.5 Hz, r'. *J*= 117.5, 5.5 Hz, s'. *J*= 17.5, 11.0 Hz, t'. *J*= 11.5, 10.5 Hz, u'. *J*= 11.0, 4.5 Hz, v'. *J*= 7.0 Hz, w'. *J*= 7.0 Hz, x'. *J*= 13.5, 12.5, 5.0 Hz, y'. *J*= 11.5, 2.0 Hz, z'. *J*= 11.5 Hz, a''. *J*= 17.0 Hz, b''. *J*= 17.0 Hz, c''. *J*= 18.0, 5.0 Hz, d''. *J*= 18.0, 12.4 Hz, e''. *J*= 11.0, 10.0, 4.5 Hz, f''. *J*= 9.8, 9.5 Hz, g''. *J*= 11.0, 4.5 Hz, h''. *J*= 7.0 Hz, i''. *J*= 7.0 Hz, j''. *J*= 13.0, 12.0, 4.5 Hz, k''. *J*= 11.5, 2.0 Hz, l''. *J*= 11.5 Hz, m''. *J*= 17.0 Hz, n''. *J*= 17.0 Hz, o''. *J*= 18.0, 5.5 Hz, p''. *J*= 18.0, 12.5 Hz, q''. *J*= 11.0, 10.0 Hz, r'. *J*= 11.0, 4.5 Hz, s''. *J*= 7.0 Hz, t''. *J*= 7.0 Hz, u''. *J*= 13.5, 13.5, 4.5 Hz, v''. *J*= 11.5 Hz, w''. *J*= 11.5 Hz, x''. *J*= 16.5 Hz, y''. *J*= 16.5 Hz, z''. *J*= 18.0, 6.0 Hz, a'''. *J*= 11.5, 4.5 Hz, b'''. *J*= 7.0 Hz, c'''. *J*= 7.0 Hz, d''. *J*= 13.0, 13.0, 5.0 Hz, e'''. *J*= 11.0, 2.0 Hz, f'''. *J*= 11.0 Hz, g'''. *J*= 17.0 Hz, h'''. *J*= 17.0 Hz, i'''. *J*= 11.0, 5.0 Hz, j'''. *J*= 11.5, 4.5 Hz, k'''. *J*= 7.0 Hz, l'''. *J*= 7.0 Hz, m'''. *J*= 13.5, 13.0, 5.0 Hz, n'''. *J*= 11.0, 2.0 Hz, o'''. *J*= 11.0 Hz,

Ritterazine J (**10**) was most highly oxygenated as revealed by a molecular formula of $C_{54}H_{76}N_2O_{11}$. The structure including stereochemistry of the western hemisphere of **10** was the same as that of **2**. The eastern hemisphere had the identical structure with the western hemisphere, except for C7 which bore no hydroxyl group.

The ^{1}H NMR spectrum of ritterazine K (**11**) was simple as compared with those of other ritterazines. It exhibited only NMR signals identical with those of the eastern hemisphere of **10**, thus suggesting a symmetrical nature. The molecular formula of $C_{54}H_{76}N_2O_{10}$ as established by HR-FABMS, was also consistent with the dimeric structure. The relative stereochemistry of **11** was the same as that of the eastern hemisphere of **10**,

which was substantiated by NOESY data. Interestingly, the structure of the steroid unit of **11** was the same as that of the western hemisphere of cephalostatin 7.[15]

Ritterazine L (**12**) had one less oxygen than ritterazine K, as revealed by HR-FABMS. The structure including stereochemistry of **12** was determined to be 17-deoxyritterazine K (**11**) on the basis of 2D NMR data.

Ritterazine M (**13**) was isomeric with **12**. Its NMR data were different from those of **12** in signals around C22; signals in the rest of the molecule were superimposable. The difference between the two compounds turned out to be the stereochemistry at C22. A ROESY cross peak between H21 and H23α suggested 22*S* stereochemistry for **13**.

Due to the paucity of material, neither absolute stereochemistry nor the orientation of the steroid units with respect to the pyrazine ring was determined for compounds **4-13**. However, it is likely that they share common structural features with ritterazines B and C, whose structures were unambiguously determined.[12]

Cytotoxic activity of ritterazines is summarized in Table IV.[30] Ritterazines B (**2**), F (**6**), and G (**7**) which have 5/5 and 5/6 spiroketal rings were most cytotoxic irrespective of C22 stereochemistry or presence of Δ^{14} olefin,[31] while oxidation of the OH-12 to a ketone (ritterazines H and I) resulted in a considerable decrease in cytotoxic activity. Ritterazines A (**1**), D (**4**), and E (**5**), which have rearranged steroid skeletons, have similar activity as the 12-keto derivatives. In these compounds stereochemistry at C22 or methylation at C24 do not affect cytotoxic activity. Ritterazine J (**10**), in which C26 is oxidized and accordingly possesses two 5/6 spiroketal groups, is less active than **6**. Since **10-13** have similar activity, the hydroxyl groups at C7 and C17 are not important for cytotoxic activity. Further investigation of the structure-activity relationships of the ritterazines is in progress.

Table IV. Cytotoxicity against P388 of all ritterazines with IC_{50} values (ng/mL)

Ritterazine A (**1**)	3.5	Ritterazine H (**8**)	16
Ritterazine B (**2**)	0.15	Ritterazine I (**9**)	14
Ritterazine C (**3**)	92	Ritterazine J (**10**)	13
Ritterazine D (**4**)	16	Ritterazine K (**11**)	9.5
Ritterazine E (**5**)	3.5	Ritterazine L (**12**)	10
Ritterazine F (**6**)	0.73	Ritterazine M (**13**)	15
Ritterazine G (**7**)	0.73	adriamycin (control)	15

Experimental Section

General Procedure. ^1H and ^{13}C NMR spectra were recorded on either a Bruker ARX-500, a Bruker AM-600, or a JEOL ALPHA-500 NMR spectrometer. Optical rotation was determined with a JASCO DIP-371 digital polarimeter. Mass spectra were measured on a JEOL SX 102 mass spectrometer. IR spectra were recorded on a JASCO FT/IR-5300 spectrophotometer. UV spectra were recorded on a Shimadzu UV-160 spectrophotometer. P388 murine leukemia cells were incubated with a TABAI BNA-111 CO_2 incubator. UV absorbance for the determination of cytotoxic activity was measured at 550 nm on a Shimadzu CS-9300PC dual wavelength flying spot scanning densitometer.

Cytotoxicity Assay. P388 murine leukemia cells (JCRB17) were cultured in RPMI 1640 medium (Nissui Pharm. Co., Tokyo) supplemented with 100 μg/mL of kanamycin (Nacalai Tesque Inc., Kyoto) , 10% of fetal bovine serum (Lot 11152276, Hyclone Laboratories, Inc., Logan, UT), and 10 μM/mL of 2-

hydroxyethyl disulfide (Nacalai Tesque Inc., Kyoto) at 37°C under an atmosphere of 5% CO_2. To each well of 96-well microplates which contained 100 μL of tumor cell suspension of 1 x 10^4 cells/mL, 100 μL of test solution (sample dissolved in RPMI 1640 medium) was added and the plates were incubated for 96 h. After addition of 50 mL of 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H tetrazolium bromide (MTT) saline solution (1 mg/mL) to each well the plates were incubated for 3 h under the same conditions. The mixtures were centrifuged and the supernatants were removed. The precipitates obtained were dissolved in DMSO, and absorbance at 550 nm was measured with a dual wavelength flying spot scanning densitometer.

Extraction and Isolation. Specimens of *Ritterella tokioka* were collected off the Izu Peninsula in August 1994 and kept frozen until processed. The thawed samples were freed from macro-epibionts, sand, and other debris before extraction. The cleaned animals (8.2 kg) were homogenized in a Waring Blendor and extracted with ethanol (10 L x 4). The combined extracts were concentrated and partitioned between water (2 L) and ethyl acetate (1.5 L x 3). The ethyl acetate-soluble portion (18.0 g) was partitioned between H_2O/MeOH (1:9) and *n*-hexane, and to the aqueous MeOH phase was added water to adjust the MeOH concentration to 60%. The mixture was extracted with CH_2Cl_2. Each fraction was monitored for cyotoxicity against P388 murine leukemia cells. The active CH_2Cl_2 layer (7.80 g) was subjected to flash chromatography on ODS (5 x 10 cm) with MeCN/H_2O (5:5), MeCN/H_2O (7:3), MeCN/H_2O (9:1), MeOH, and MeOH/$CHCl_3$/H_2O (7:3:0.5). Fraction eluted with MeCN/H_2O (7:3) (1.424 g) was gel-filtered on Sephadex LH-20 (6 x 80 cm) with C_6H_{14}/CH_2Cl_2/MeOH (4:5:1). The active fractions were combined and purified by ODS-HPLC (2 x 25 cm) with MeCN/H_2O (6:4) to give ritterazines D (**4**), E (**5**), F (**6**), G (**7**), H (**8**), I (**9**), J (**10**), K (**11**), L (**12**), and M (**13**) (yields, 4.0, 2.8, 2.6, 2.2, 1.2, 4.4, 2.8, 6.2, 1.1, and 1.9 mg, respectively) as colorless glassy solids.

4: $[\alpha]_D$ +81.4° (*c* 0.1, MeOH); UV (MeOH) λ_{max} 286 (ε 9200), and 304 (sh) nm; IR (film) 3460, 2980, 2940, 2890, 2370, 2340, 1740, 1460, 1400, 1130, 1040, 1000, and 900 cm^{-1}; HR-FABMS (positive) *m/z* 913.5633 ($C_{54}H_{77}N_2O_{10}$, Δ +5.5 mmu); ^{13}C NMR data in pyridine-*d5* at 300 K, see Table I; ^1H NMR data in pyridine-*d5* at 293 K, see Tables II and III.

5: $[\alpha]_D$ +70.8° (*c* 0.1, MeOH); UV (MeOH) λ_{max} 288 (ε 10100), and 307 (sh) nm; IR (film) 3440, 2960, 2920, 2870, 2350, 2320, 1730, 1710, 1600, 1460, 1400, 1140, 1030, 960, and 880 cm^{-1}; HR-FABMS (positive) *m/z* 927.5724 ($C_{55}H_{79}N_2O_{10}$, Δ -1.1 mmu); ^{13}C NMR data in pyridine-*d5* at 300 K, see Table I; ^1H NMR data in pyridine-*d5* at 293 K, see Tables II and III.

6: $[\alpha]_D$ +59.0° (*c* 0.1, MeOH); UV (MeOH) λ_{max} 288 (ε 7910), and 306 (sh) nm; IR (film) 3470, 2960, 2920, 2860, 2350, 2340, 1700, 1600, 1460, 1400, 1220, 1140, 1040, and 880 cm^{-1}; HR-FABMS (positive) *m/z* 899.5764 ($C_{54}H_{79}N_2O_9$, Δ -2.1 mmu); ^{13}C NMR data in pyridine-*d5* at 300 K, see Table I; ^1H NMR data in pyridine-*d5* at 293 K, see Tables II and III.

7: $[\alpha]_D$ +91.4° (*c* 0.1, MeOH); UV (MeOH) λ_{max} 288 (ε 11200), and 306 (sh) nm; IR (film) 3450, 2960, 2930, 2870, 2360, 2340, 1730, 1600, 1450, 1400, 1230, 1140, 1040, 1000, and 880 cm^{-1}; HR-FABMS (positive) *m/z* 897.5598 ($C_{54}H_{77}N_2O_9$, Δ -3.1 mmu); ^{13}C NMR data in pyridine-*d5* at 300 K, see Table I; ^1H NMR data in pyridine-*d5* at 293 K, see Tables II and III.

8: $[\alpha]_D$ +96.0° (*c* 0.1, MeOH); UV (MeOH) λ_{max} 287 (ε 8920), and 306 (sh) nm; IR (film) 3460, 2960, 2920, 2860, 2350, 2320, 1700, 1590, 1450, 1400, 1360, 1230, 1130, 1040, and 880 cm^{-1}; HR-FABMS (positive) *m/z* 897.5591 ($C_{54}H_{77}N_2O_9$, Δ -3.8 mmu); ^{13}C NMR data in pyridine-*d5* at 300 K, see Table I; ^1H NMR data in pyridine-*d5* at 293 K, see Tables II and III.

9: $[\alpha]_D$ +74.5° (*c* 0.1, MeOH); UV (MeOH) λ_{max} 286 (ε 9120), and 306 (sh) nm; IR (film) 3460, 2960, 2920, 2860, 1700, 1450, 1400, 1300, 1230, 1110, 1040, and 880 cm^{-1}; HR-FABMS (positive) *m/z* 913.5663 ($C_{54}H_{77}N_2O_{10}$, Δ +8.5 mmu); ^{13}C NMR data in pyridine-d_5 at 300 K, see Table I; ^1H NMR data in pyridine-d_5 at 293 K, see Tables II and III.

10: $[\alpha]_D$ +66.1° (*c* 0.1, MeOH); UV (MeOH) λ_{max} 289 (ε 8420), and 308 (sh) nm; IR (film) 3450, 2960, 2920, 2880, 2360, 2320, 1730, 1700, 1450, 1400, 1300, 1230, 1110, 1060, 1040, 990, 970, 940, 880, and 850 cm^{-1}; HR-FABMS (positive) *m/z* 929.5471 ($C_{54}H_{77}N_2O_{11}$, Δ -5.6 mmu); ^{13}C NMR data in pyridine-d_5 at 300 K, see Table I; ^1H NMR data in pyridine-d_5 at 293 K, see Tables II and III.

11: $[\alpha]_D$ +74.0° (*c* 0.1, MeOH); UV (MeOH) λ_{max} 288 (ε 7100), and 306 (sh) nm; IR (film) 3480, 2960, 2930, 2870, 2360, 2320, 1700, 1450, 1400, 1300, 1230, 1200, 1140, 1110, 1060, 1040, 990, 960, 940, 880, and 850 cm^{-1}; HR-FABMS (positive) *m/z* 913.5532 ($C_{54}H_{77}N_2O_{10}$, Δ -4.7 mmu); ^{13}C NMR data in pyridine-d_5 at 300 K, see Table I; ^1H NMR data in pyridine-d_5 at 293 K, see Table II.

12: $[\alpha]_D$ +85.5° (*c* 0.1, MeOH); UV (MeOH) λ_{max} 288 (ε 11000), and 307 (sh) nm; IR (film) 3440, 2960, 2920, 2860, 2360, 2320, 1600, 1450, 1400, 1230, 1040, 940, 880, and 850 cm^{-1}; HR-FABMS (positive) *m/z* 897.5598 ($C_{54}H_{77}N_2O_9$, Δ -3.1 mmu); ^{13}C NMR data in pyridine-d_5 at 300 K, ssee Table I; ^1H NMR data in pyridine-d_5 at 293 K, see Tables II and III.

13: $[\alpha]_D$ +95.1° (*c* 0.1, MeOH); UV (MeOH) λ_{max} 289 (ε 11900), and 306 (sh) nm; IR (film) 3460, 2960, 2920, 2860, 2360, 2300, 1700, 1460, 1400, 1040, 940, 880, and 850 cm^{-1}; HR-FABMS (positive) *m/z* 897.5591 ($C_{54}H_{77}N_2O_9$, Δ -3.8 mmu); ^{13}C NMR data in pyridine-d_5 at 300 K, see Table I; ^1H NMR data in pyridine-d_5 at 293 K, see Tables II and III.

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30. IC_{50} values fluctuated to some degree depending on the condition of the cells used. For comparison sake the values in Table IV were determined by using cells grown at the same time. The IC_{50} values for **1**-**3** are higher than those reported by one order of magnitude.
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