Isolation and Structure Elucidation of Ritterazines B and C, Highly Cytotoxic Dimeric Steroidal Alkaloids, from the Tunicate *Ritterella tokioka*[1]

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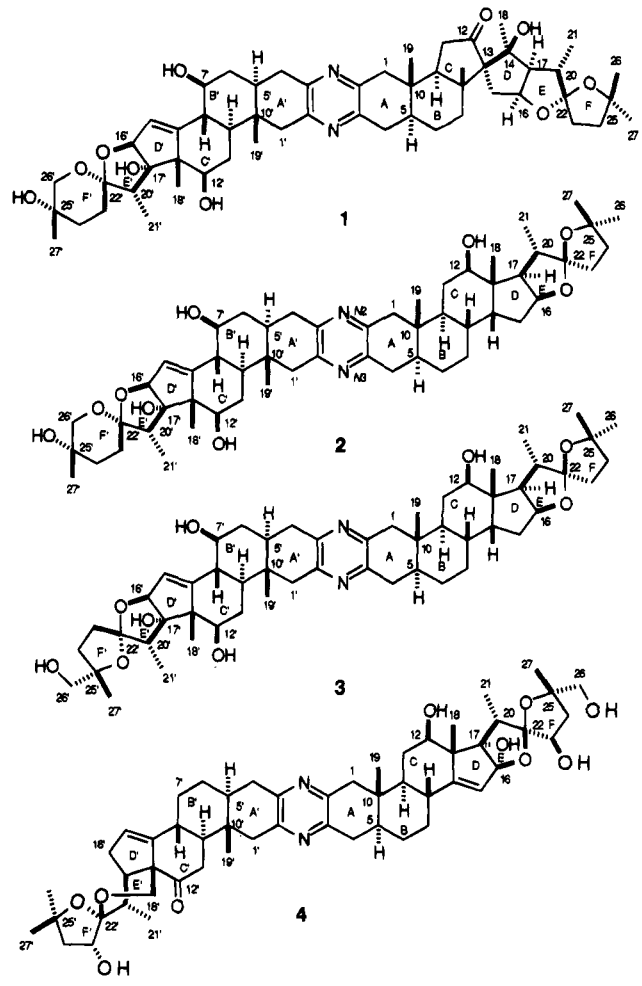
Ritterazines B and C, dimeric steroidal alkaloids related to the cephalostatins, have been isolated from the tunicate *Ritterella tokioka* and their structures including absolute stereochemistry have been elucidated by spectral and chemical methods. Ritterazines B and C showed potent cytotoxicity against the P388 murine leukemia cells with IC_{50}'s of 0.018 and 9.4 ng/mL, respectively.

Tunicates have proven to be a potential source of anticancer drugs as represented by the didemnins and ecteinascidins. In addition, highly cytotoxic metabolites such as the eudistomins, patellazoles, ulithiacyclamides, varamines, iejimalides, and diazonamides have been isolated.[2] In our continuning 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 three highly bioactive compounds, ritterazines A (**1**), B (**2**), and C (**3**). We have already reported the structure of ritterazine A,[3] which is a dimeric steroidal alkaloid related to the cephalostatins[4] isolated from the hemichordate *Cephalodiscus gilchristi*. In this paper, we report the isolation and structure elucidation of ritterazines B and C.

Colonies of the tunicate[5] (5.5 kg) were extracted with EtOH and then with acetone. The combined extracts were concentrated and partitioned between water and ethyl acetate. The organic phase was fractionated by the Kupchan partitioning procedure;[6] most of the cytotoxicity against the P388 murine leukemia cells was found in the CH_2Cl_2 phase. The CH_2Cl_2 soluble materials were repeatedly purified by ODS and Sephadex LH-20 chromatographies to yield ritterazines B (**2**) and C (**3**) (13.4 and 7.8 mg, respectively) as colorless glassy solids. Ritterazines B and C showed cytotoxicity against the P388 murine leukemia cells[7] with IC_{50} values of 0.018 and 9.4 ng/mL, respectively.



Ritterazine B (**2**) showed an $(M + H)^+$ ion at m/z 899.5873 in HR-FABMS, matching a molecular formula of $C_{54}H_{78}N_2O_9$ (Δ +8.7 mmu). The UV spectrum[8] [λ_{max} 288 nm (ϵ 6880)] suggested the presence of a pyrazine as found in ritterazine A, which was substantiated by

[⊗] Abstract published in *Advance ACS Abstracts,* January 1, 1995.

(1) Bioactive Marine Metabolites. 68. Part 67: Ryu, G.; Matsunaga, S.; Fusetani, N. *Tetrahedron* **1994**, *50*, 13409–13416.

(2) Davidson, B. S. *Chem. Rev.* **1993**, *93*, 1771–1791.

(3) Fukuzawa, S.; Matsunaga, S.; Fusetani, N. *J. Org. Chem.* **1994**, *59*, 6164–6166.

(4) (a) Pettit, G. R.; Inoue M.; Kamano, Y.; Herald, D. L.; Arm, C.; Dufresne, C.; Christie, N. D.; Schmidt, J. M.; Doubek, D. L.; Krupa, T. S. *J. Am. Chem. Soc.* **1988**, *110*, 2006–2007. (b) Pettit, G. R.; Inoue M.; Kamano, Y.; Dufresne, C.; Christie, N. D.; Niven, M. L.;Herald, D. L. *J. Chem. Soc., Chem. Commun.* **1988**, 865–867. (c) Pettit, G. R.; Kamano, Y.; Dufresne, C.; Inoue M.; Christie, N. D.; Jean, M. *Can. J. Chem.* **1989**, *67*, 1509–1513. (d) Pettit, G. R.; Kamano, Y.; Inoue. M.; Dufresne, C.; Boyd, M. R.; Herald, D. L.; Schmidt, J. M.; Doubek, D. L.; Christie, N. D. *J. Org. Chem.* **1992**, *57*, 429–431. (e) Pettit, G. R.; Ichihara, Y.; Xu, J. P.; Williams, M. D.; Christie, N. D.; Doubek, D. L.; Schmidt, J. M.; Boyd, M. R. *J. Nat. Prod.* **1994**, *57*, 52–63. (f) Pettit, G. R.; Ichihara, Y.; Xu, J.; Boyd, M. R.; Williams, M. D. *Bioorg. Med. Chem. Lett.* **1994**, *4*, 1507–1512.

(5) Colonies were collected by hand using scuba at depths of 2–10 m off the Izu Peninsula, 100 km southwest of Tokyo. They were identified as *Ritterella tokioka* Kott, 1992 (family Polyclinidae, order Enterogona) by Dr. T. Nishikawa (Nagoya University). The voucher specimen (T94-001) was deposited at the Laboratory of Marine Biochemistry, The University of Tokyo.

(6) Kupchan, S. M.; Britton, R. W.; Ziegler, M. F.; Sigel, C. W. *J. Org. Chem.* **1973**, *38*, 178–179.

(7) Inaba, M.; Nagashima, K. *Jpn. J. Cancer Res.* **1986**, *77*, 197–204.

(8) Pretsch, E.; Simon, W.; Seibl. J.; Clerc, T. In *Tables of Spectral Data for Structure Determination of Organic Compounds*, 2nd Engl. ed.; Fresenius, W., Huber, J. F. K., Pungor, E., Rechnitz, G. A., Simon, W., West, Th. S., Eds.; Springer-Verlag: Berlin, 1989; pp U5–U155.

0022-3263/95/1960-0608$09.00/0

Table 1. ^1H and ^{13}C NMR Data of Ritterazine B (pyridine-*d5*)

left side				right side			
no.	^{13}C (ppm)	^1H (ppm)	*J* values (Hz)	no.	^{13}C (ppm)	^1H (ppm)	*J* values (Hz)
1′	45.9 t	α 2.68 d	18.3	1	46.3 t	α 2.71 d	18.0
		β 3.15 d	18.3			β 3.17 d	18.0
2′	148.6 s			2	149.3 s		
3′	148.1 s			3	149.0 s		
4′	35.6 t	α 2.98 dd	17.7, 5.2	4	35.9 t	α 2.94 dd	17.5, 5.2
		β 2.77 dd	17.7, 12.5			β 2.68 dd	17.5,13.1
5′	40.0 d	1.84 m		5	41.5 d	1.57 m	
6′	38.4 t	α 2.18 dd	12.4, 4.4	6	29.0 t	α 1.48 m	
		β 1.76 ddd	12.4, 12.3, 10.5			β 1.28 m	
7′	69.4 d	4.06 dddd	10.6, 10.5, 4.4, 2.2	7	31.7 t	α 1.10 m	
						β 1.49 m	
8′	42.8 d	2.41 dd	10.6, 10.0	8	32.6 d	1.68 m	
9′	51.2 d	1.16 m		9	45.5 d	1.36 m	
10′	35.9 s			10	35.9 s		
11′	29.2 t	α 2.17 m		11	30.7 t	α 2.04 m	
		β 1.88 m				β 1.67 m	
12′	75.6 d	4.20 ddd	11.2, 4.6, 2.0	12	71.8 d	3.64 dd	11.7, 3.7
13′	56.1 s			13	48.6 s		
14′	151.6 s			14	47.8 d	2.08 m	
15′	121.1 d	6.13 d	2.0	15	32.8 t	α 1.80 dd	15.1, 13.8
						β 1.83 dd	15.1, 7.0
16′	94.0 d	5.25 d	2.0	16	80.0 d	4.78 dd	9.7, 7.0
17′	93.3 s			17	57.5 d	3.15 dd	9.7, 9.6
18′	12.5 q	1.33 s		18	13.7 q	1.26 s	
19′	11.5 q	0.85 s		19	11.9 q	0.75 s	
20′	48.2 d	2.21 d	6.9	20	42.0 d	2.01 dq	9.6, 6.7
21′	8.1 q	1.26 q	6.9	21	14.7 q	1.18 d	6.7
22′	107.9 s			22	117.0 s		
23′	27.5 t	α 2.50 ddd	13.4, 13.4, 4.8	23	33.2 t	α 1.85 m	
		β 1.49 m				β 2.12 m	
24′	33.2 t	α 1.87 m		24	37.3 t	α 1.68 m	
		β 2.16 m				β 2.04 m	
25′	65.8 s			25	81.4 s		
26′	70.2 t	α 3.61 dd	11.6, 2.7	26	28.8 q	1.18 s	
		β 4.02 d	11.6				
27′	27.0 q	1.22 s		27	30.8 q	1.43 s	
7′-OH		3.63 d	2.2	12-OH		5.80 br s	
12′-OH		4.67 d	2.0				
17′-OH		5.00 s					
25′-OH		3.69 br s					

^{13}C NMR signals at δ 148.1, 148.6, 149.0, and 149.3.[9] ^{13}C NMR data implied the presence of nine methyls, 15 methylenes, 16 methines, and 14 quarternary carbons (Table 1), indicating that ritterazine B was related to the cephalostatins [cephalostatin 1 (**4**)].[4a]

The presence of a trisubstituted olefin reminiscent of ritterazine A (**1**)[3] was deduced by ^{13}C NMR data (δ 151.6 s, 121.2 d), but unlike the spectral feature of **1**, no ketone signal was observed. Partial structures **a**–**e** were derived by interpretation of COSY and HMQC spectra[10] and from analysis of the methyl proton region of the HMBC spectrum (Figure 1).[11] Connectivities of these partial structures were established on the basis of HMBC data (Table 2), thereby leading to two polyoxygenated steroidal halves fused via a pyrazine at C2 and C3,[12] which was consistent with ^1H NMR data [H1 (δ 2.71, 3.17), H4 (δ 2.68, 2.94), H1′ (δ 2.64, 3.15), and H4′ (δ 2.77, 2.98)]. The orientation of the steroidal nuclei with respect to the pyrazine ring could not be determined by NMR spectroscopy. Three primary or secondary hydroxyl groups were readily confirmed by formation of a triacetate upon treatment with Ac_2O in pyridine. Conversely, one of the three hydroxyls in retterazine A (**1**) is tertiary.[3]

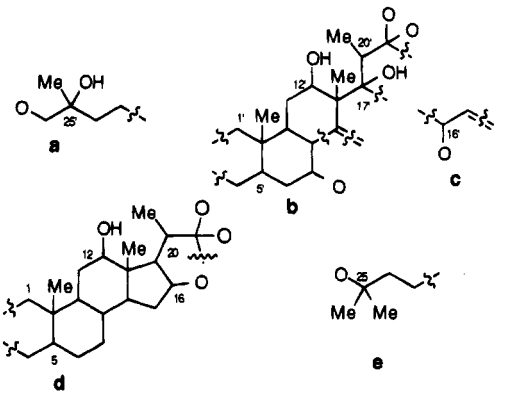


Figure 1. Partial structures of ritterazine B (**2**).

The relative stereochemistry of the two steroidal units in **2** was determined by NOESY data together with values of coupling constants (Figure 2). The ^1H and ^{13}C NMR signals of the western hemisphere of **2** were almost superimposable on ritterazine A (**1**), thus revealing that the western hemisphere of ritterazine B had the same gross structure as that of ritterazine A. Furthermore, the NOESY spectrum of **2** exhibited the same sets of cross peaks that were observed for the western hemisphere of **1**; thus, both western hemispheres had identical relative stereochemistry. The eastern hemisphere of ritterazine B (**2**) contained a saturated ring D, a hydroxyl group at C12, and a 5/5 spiroketal system. Axial orientation of

(9) Breitmaier, E.; Voelter, W. In *Carbon 13 NMR Spectroscopy*, 3rd ed.; VCH: New York, 1990; pp 281–286.
(10) Summers, M. F.; Marzilli, L. G.; Bax, A. *J. Am. Chem. Soc.* **1986**, *108*, 4285–4294.
(11) Bax, A.; Azolos, A.; Dinya, Z.; Sudo, K. *J. Am. Chem. Soc.* **1986**, *108*, 8056–8063.
(12) Assignments for C2, C3, C2′, and C3′ may be interchanged.

Table 2. NOESY and HMBC Data of Ritterazine B

left side				right side			
no.	δ	NOE	HMBC	no.	δ	NOE	HMBC
H-1′α	2.68	H1′β, H9′	C2′, C9′, C10′, C19′	H-1α	2.71	H1β, H9	C2, C5, C9, C10, C19
H-1′β	3.15	H1′α, H11′α, H11′β	C2′, C5′, C10′	H-1β	3.17	H1α, H11α, H11β	C2, C5, C10, C19
H-4′α	2.98	H4′β, H5′, H6′α, H6′β	C3′, C5′, C10′	H-4α	2.94	H4β, H5, H6α, H6β	C3, C5, C10
H-4′β	2.77	H4′α, H6′β	C3′, C5′	H-4β	2.68	H4α, H6β	C3, C5, C10
H-5′	1.84	H4′α, H6′α, H7′, H9′		H-5	1.57	H4α, H7α, H9	
H-6′α	2.18	H4′α, H5′, H6′β, H7′		H-6α	1.48	H4α, H6β, H7α	
H-6′β	1.76	H4′α, H4′β, H6′α, H7′, H8′	C8′	H-6β	1.28	H4α, H4β, H6α	
H-7′	4.06	H5′, H6′α, H6′β, H9′,		H-7α	1.10	H5, H6α, H15β	
7′-OH	3.63			H-7β	1.49	H8, H14	
H-8′	2.41	H6′β,, H19′	C7′, C9′, C14′, C15′	H-8	1.68	H7β, H18, H19	C9
H-9′	1.16	H1′α, H5′, H7′, H12′		H-9	1.36	H1α, H5, H12, H15α	
H-11′α	2.17	H1′β, H11′β, H12′	C9′, C12′, C13′	H-11α	2.04	H1α, H1β, H11β,H12	C13
H-11′β	1.88	H1′β, H11′α, H18′, H19′	C9′, C12′, C13′	H-11β	1.67	H1β, H11α, H19	C9, C12
H-12′	4.20	H9′, H11′α, H16′,17′-OH	C17′, C18′	H-12	3.64	H9, H11α, H16, H17	
12′-OH	4.67	17′-OH	C11′	12-OH	5.80		
				H-14	2.08	H7β	C13
H-15′	6.13	H7′, H24′β	C8′, C13′, C14′, C16′, C17′	H-15α	1.83	H16, H9	C8, C14
				H-15β	1.80	H7α	C13, C14, C16, C17
H-16′	5.25	H12′, H26′β	C14′, C17′	H-16	4.78	H12, H15α, H17	C13, C14
17′-OH	5.00	H12′. 12′-OH	C13′, C17′	H-17	3.15	H12, H16, H21	C12, C13, C14, C15, C20, C21
H-18′	1.33	H11′β, H20′	C12′, C13′, C14′, C17′	H-18	1.26	H8, H20	C12, C13, C14, C17
H-19′	0.85	H8′, H11′β	C1′, C5′, C9′, C10′	H-19	0.75	H8, H11β	C1, C5, C9, C10
H-20′	2.21	H18′, H23′α, H23′β	C17′, C13′, C21′, C22′, C23′	H-20	2.01	H18, H23α	C13, C17, C21, C23
H-21′	1.26		C17′, C20′, C22′	H-21	1.18	H17	C17, C20, C22
H-23′α	2.50	H20′, H23′β	C22′, C24′	H-23α	1.85		
H-23′β	1.49	H20′, H23′α, H24′β		H-23β	2.12	H20	
H-24′α	1.87	H15′, H24′β, H23′β		H-24α	1.68	H26	C22
H-24′β	2.16	H24′α	C23′	H-24β	2.04		C22
25′-OH	3.69		C26′				
H-26′α	3.61	H16′, H26′β	C22′, C24′, C25′	H-26	1.18	H24α	C24, C25, C27
H-26′β	4.02	H16′, H26′α, H27′	C22′, C25′				
H-27′	1.22	H26′β	C24′, C25′, C26′	H-27	1.43	H23β	C24, C25, C26

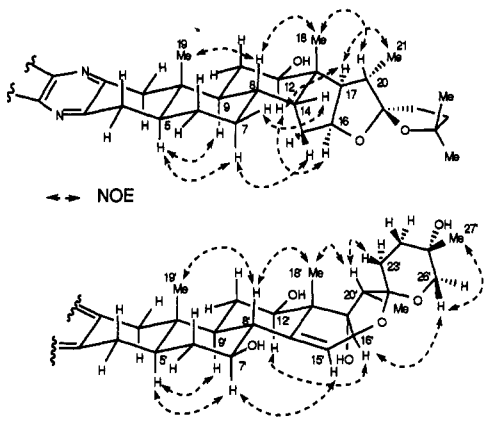


Figure 2. NOESY data of the eastern (upper) and western (lower) hemispheres of ritterazine B (**2**).

H5, H8, H9, and H12 was evident from large vicinal coupling constants.[13] Although H8 and H11β signals overlapped hampering interpretation of NOESY cross peaks between CH_3-19 and these signals, *trans*-fusion of rings A/B could be deduced on the basis of the ^{13}C chemical shift of C19 at 11.9 ppm.[14] A NOESY cross peak observed between H14 and H7β implied *cis*-fusion for rings C/D, which was supported by an additional NOESY cross peak for H15β/H7α. In fact, chemical shifts of C9 and C14 significantly differed from those for hippuristanol,[15] which has an analogous steroidal skeleton with C/D *trans* fusion. Similarly, NOESY cross peaks H12/H16, H12/H17, CH_3-18/H20, and CH_3-21/H17 allowed assignment of the relative stereochemistry in rings D and E. Assignment of the stereochemistry at C22 was done by measuring the NOESY spectrum in CD_3OD at 263 K, which gave a cross peak between CH_3-21 and H23β, suggesting the C22*R* stereochemistry.[16]

In order to obtain *N*-methyl derivatives, which would allow for the determination of the orientation of the steroidal units about the pyrazine ring, ritterazine B was treated with MeI to afford a reaction mixture generating four HPLC peaks. These products proved to be either *N*2- or *N*3-methyl derivatives of 5/5 or 6/5 spiroketals at C22′ as judged from ^1H NMR data. In fact, the first two peaks corresponded to *N*3-methyl ritterazine B (**5**) and *N*3-methyl ritterazine C (**6**), respectively, while the other two were *N*2-methyl ritterazine B (**7**) and *N*2-methyl ritterazine C (**8**), indicating that isomerization at C22′ took place during HPLC workup. Fortunately, the chemical shifts of protons in rings A and A′ were not affected by the isomerization at C22′. The mixture of **5** and **6** exhibited NOESY cross peaks between *N*3-Me/H1′α, H1′β, H4α, and H4β, while the mixture of **7** and **8** showed NOESY cross peaks *N*2-Me/H1α, H1β, H4′α, and H4′β. Therefore, the orientation of the two steroidal units in ritterazines B and C was identical with that of cephalostatin 1 (**4**)[4a] whose structure was established by X-ray diffraction.

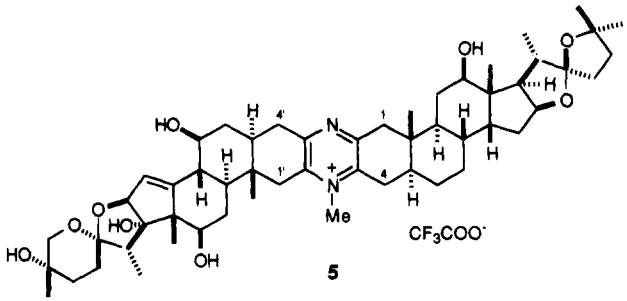
Ritterazine C (**3**) was an isomer of **2**. The ^1H NMR spectrum of **3** displayed the same sets of signals observed

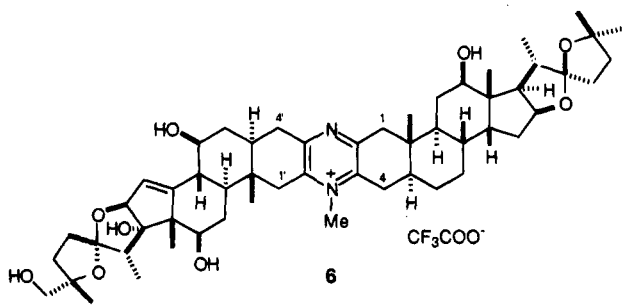
(13) A large (ca. 12 Hz) coupling between H8 and H9 was determined by the DQF-COSY spectrum.

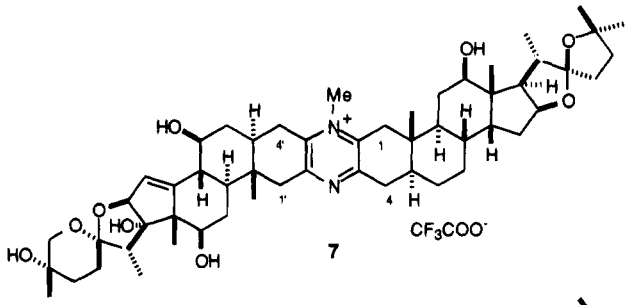
(14) Blunt, J. W.; Stothers, J. B. *Org. Magn. Reson.* **1977**, *9*, 439–464.

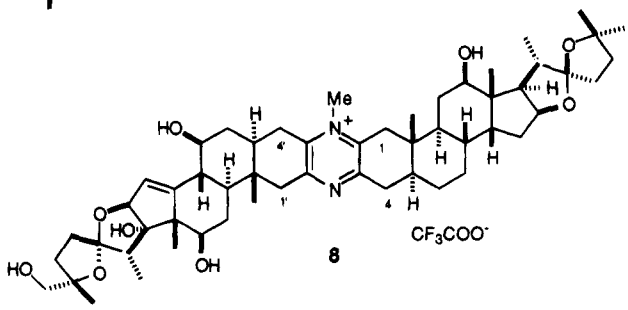
(15) Higa, T.; Tanaka, J.; Tsukitani, Y.; Kikuchi, H. *Chem. Lett.* **1981**, 1647–1650.

(16) In the NOESY spectra of **2** in pyridine-d_5 at 300 K, no cross peak was observed for protons in ring F, indicating an unfavorable correlation time for this part of the molecule. This problem was circumvented by measurement of the spectrum at 273 K. However, an unambiguous assignment of cross peaks between protons in rings E and F was not accomplished due to signal overlapping of H20, H23, and H24.









in the eastern hemisphere of ritterazine B; the rest of the spectrum was different from the western hemisphere of **2** (Tables 3 and 4). Interpretation of COSY, HMQC, and HMBC data and the chemical shift of C22 (δ 118.1) suggested the presence of a 5/5 spiroketal in the western hemisphere (Figure 3). Ritterazine B was converted to a 1:1 mixture of ritterazines B and C when kept in $CDCl_3$ solution overnight.[17] Therefore, the stereochemistry of **3** except for C22 must be identical with that of **2**. The stereochemistry at C22 is likely to be identical in **2** and **3**. It should be noted that a similar equilibration between 5/5 and 5/6 spiroketal systems has been reported during synthetic approaches to cephalostatins.[18]

Determination of the absolute configuration of ritterazine C was attempted by application of the modified Mosher method.[19] Ritterazine C was treated with (*S*)- and (*R*)-MTPACl in pyridine to yield the corresponding tris-MTPA esters.[20] The distribution of the positive and negative $\Delta\delta$ ($\delta_{(-)S} - \delta_{(+)R}$) values around the MTPA ester groups was in agreement with C7′-*S* and C12-*R* stereochemistry (Figure 4). Therefore, the two steroidal units had absolute stereochemistry identical with conventional steroids. This is the first determination of the absolute configuration of this class of compounds, although the Pettit group speculated about the absolute configuration of cephalostatins from X-ray data.[4a]

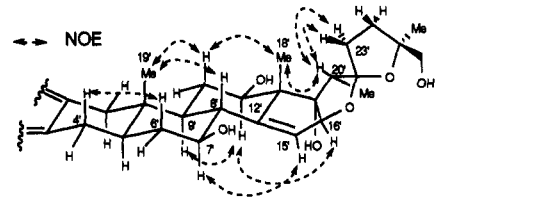


Figure 3. NOESY data of the western hemisphere of ritterazine C (**3**).

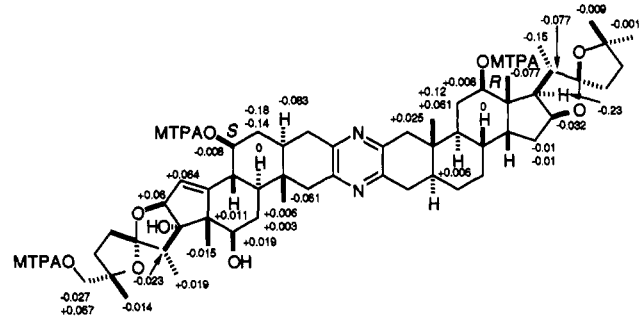


Figure 4. [$\Delta\delta = \delta_{S(-)} - \delta_{R(+)}$] values obtained for ritterazine C MTPA esters.

The ritterazines and cephalostatins share the common structural features in which two highly oxygenated hexacyclic steroidal units are fused via a pyrazine ring at C2 and C3 and both side chains of steroidal units form either 5/5 or 5/6 spiroketals. The cephalostatins have the more oxygenated eastern hemispheres than the ritterazines, while the western hemispheres are more oxygenated in the ritterazines than the cephalostatins; hydroxyl groups are seen at C12, C17, C23, C26, C12′, and C23′ in the cephalostatins, whereas C12, C7′, C12′, C17′, and C25′ are hydroxylated in the ritterazines. Interestingly, the cephalostatins are much more cytotoxic against the P388 murine leukemia cells ($IC_{50} = 10^{-4} - 10^{-7}$ ng/mL) than the ritterazines ($IC_{50} \approx 9.4 - 0.018$ ng/mL).

Ritterazines are remarkably cyotoxic tunicate metabolites closely related to cephalostatins isolated from an Indian Ocean hemichordate. Occurrence of these compounds in different phyla may indicate a microbial origin of the cephalostatin class of compounds. Discovery of the ritterazines will stimulate research on the mode of action of this important class of compounds as well as development of new anticancer drugs.

Experimental Section

General Procedure. ^1H and ^{13}C NMR spectra were recorded on either a Bruker AM-600 or a JEOL ALPHA-

(17) The isomerization did not take place by standing **2** in $CHCl_3$ which contained a trace amount of EtOH as a stabilizer, indicating that the isomerization was catalyzed by a trace amount of DCl in $CDCl_3$. We did not employ acidic or basic media during the isolation procedure of **2** and **3**. Therefore, it is likely that both ritterazines B and C are natural products.

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(20) At present we have no idea of why the hydroxyl group on C12′ was resistant to esterification with MTPA.

Table 3. ^1H and ^{13}C NMR Data of Ritterazine C (pyridine-d_5)

left side				right side			
no.	^{13}C (ppm)	^1H (ppm)	J values (Hz)	no.	^{13}C (ppm)	^1H (ppm)	J values (Hz)
1′	45.8 t	α 2.64 d	17.1	1	46.3 t	α 2.72 d	16.6
		β 3.11 d	17.1			β 3.16 d	16.6
2′	148.5 s			2	149.4 s		
3′	148.3 s			3	148.8 s		
4′	35.6 t	α 2.96 dd	17.7, 5.2	4	35.8 t	α 2.95 dd	18.1, 3.6
		β 2.75 dd	17.7, 12.1			β 2.66 dd	18.1, 12.9
5′	40.0 d	1.81 m		5	41.5 d	1.56 m	
6′	38.4 t	α 2.17 m		6	29.0 t	α 1.47 m	
		β 1.72 m				β 1.29 m	
7′	69.4 d	4.00 ddd	10.6, 10.1, 4.6	7	31.8 t	α 1.11 m	
						β 1.49 m	
8′	42.7 d	2.40 dd	10.6, 10.2	8	32.6 d	1.65 m	
9′	51.3 d	1.15 m		9	45.5 d	1.36 m	
10′	35.8 s			10	35.8 s		
11′	29.4 t	α 2.16 m		11	30.8 t	α 2.04 m	
		β 1.87 m				β 1.68 m	
12′	75.7 d	4.16 dd	11.3, 4.8	12	71.8 d	3.64 dd	9.5, 4.4
13′	55.9 s			13	48.6 s		
14′	151.5 s			14	47.8 d	2.08 m	
15′	121.3 d	5.98 dd	1.7, 1.6	15	32.8 t	α 1.80 m	
						β 1.84 m	
16′	93.4 d	5.25 d	1.7	16	80.0 d	4.77 dd	7.0, 7.0
17′	92.9 s			17	57.5 d	3.15 m	
18′	12.6 q	1.35 s		18	13.7 q	1.26 s	
19′	11.8 q	0.83 s		19	11.9 q	0.75 s	
20′	45.0 d	2.34 m		20	42.0 d	2.03 m	
21′	8.2 q	1.19 d	7.0	21	14.7 q	1.17 d	6.6
22′	118.1 s			22	117.0 s		
23′	32.1 t	α 2.36 m		23	33.2 t	α 1.70 m	
		β 1.65 m				β 2.12 m	
24′	33.5 t	α 2.02 m		24	37.8 t	α 1.67 m	
		β 1.67 m				β 2.02 m	
25′	86.1 s			25	81.4 s		
26′	69.7 t	a 3.80 d	10.8	26	28.8 q	1.19 s	
		b 3.76 d	10.8				
27′	23.7 q	1.29 s		27	30.3 q	1.43 s	
7′-OH		3.62 br s		12-OH		3.63 br s	

Table 4. NOESY and HMBC Data of Ritterazine C

left side				right side			
no.	δ	NOE	HMBC	no.	δ	NOE	HMBC
H-1′α	2.64	H1′β, H9′, H11′α	C2′, C9′, C10′, C19′	H-1α	2.72	H1β, H9	C2, C10, C19
H-1′β	3.11	H1′α, H11′α	C2′, C5′, C10′, C19′	H-1β	3.16	H1α, H11α	C2, C5, C10, C19
H-4′α	2.96	H4′β, H5′, H6′α	C3′, C5′, C10′	H-4α	2.95	H4β, H5, H6α	C3, C5, C10
H-4′β	2.75	H4′α, H6′β	C3′, C5′	H-4β	2.66	H4α, H6β	C3, C5
H-5′	1.81	H4′α, H7′		H-5	1.56	H4α, H7α, H9	
H-6′α	2.17	H4′α		H-6α	1.47	H4α, H6β, H7α	
H-6′β	1.72	H4′β	C5′, C7′	H-6β	1.29	H4β, H6α, H8	
H-7′	4.00	H5′, H11′α, H15′		H-7α	1.11	H5, H6α, H15α	
7′-OH	3.62			H-7β	1.49		
H-8′	2.40	H11′β, H19′	C7′, C9′, C14′, C15′	H-8	1.65	H6β, H18, H19	C9
H-9′	1.15	H1′α, H12′	C10′, C19′	H-9	1.36	H1α, H5, H15β	
H-11′α	2.16	H1′α, H1′β, H7′, H11′β, H12′	C9′, C12′, C13′	H-11α	2.04	H1β, H12	
H-11′β	1.87	H8′, H11′α, H18′, H19′	C9′, C12′	H-11β	1.68		
H-12′	4.16	H9′, H11′α, H16′	C17′, C18′	H-12	3.64	H11α, H16, H17	
				12-OH	3.63		C12
				H-14	2.08		
H-15′	5.98	H7′	C8′, C13′, C14′, C16′, C17′	H-15α	1.80	H7α	C13, C14, C16, C17
				H-15β	1.84	H9, H16	C14
H-16′	5.25	H12′	C14′, C17′	H-16	4.77	H12, H15β, H17	C14
				H-17	3.15	H12, H16, H21	C12, C14, C15, C21
H-18′	1.35	H11′β, H20′	C12′, C13′, C14′, C17′	H-18	1.26	H8, H20	C12, C13, C14, C17
H-19′	0.83	H8′, H11′β	C1′, C5′, C9′, C10′	H-19	0.75	H8	C1, C5, C9, C10
H-20′	2.34	H18′, H21′, H23′α, H23′β	C13′, C17′, C21′, C22′	H-20	2.03	H18, H21	C13, C17, C21, C22, C23
H-21′	1.19	H20′	C17′, C20′, C22′	H-21	1.17	H17, H20	C17, C20, C22
H-23′α	2.36	H20′, H23′β		H-23α	1.70	H23β	
H-23′β	1.65	H20′, H23′α	C24′	H-23β	2.12	H23α	
H-24′α	2.02	H24′β		H-24α	1.67	H24β	
H-24′β	1.67	H24′α		H-24β	2.02	H24α	
H-26′a	3.80		C24′, C25′, C27′	H-26	1.19		C24, C25, C27
H-26′b	3.76		C24′, C25′, C27′				
H-27′	1.29		C24′, C25′, C26′	H-27	1.43		C24, C25, C26

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. The P388 murine leukemia

cells were incubated with a TABAI BNA-111 CO_2 incubator. UV absorbance was measured at 550 nm on a CORONA MTP-32 micro plate reader.

Cytotoxicity Assay. The 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 42H0342, Sigma Chemical Co., St. Louis, MO), and 10 μM/mL of 2-hydroxyethyl disulfide (Nacalai Tesque Inc., Kyoto) at 37 °C under the atomosphere of 5% CO_2. To each well of 96-well micro plates which contained 100 μL of a tumor cell suspension of 1 × 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 μL of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-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 micro plate reader.

Extraction and Isolation. Specimens of *Ritterella tokioka* were collected off the Izu Peninsula and kept frozen until processed. The thawed samples were freed from macroepibionts, sand, and other debris before extraction. The cleaned animals (5.5 kg) were homogenized in a Waring Blendor and extracted with ethanol (5 L × 2) and then acetone (5 L). The combined extracts were concentrated and partioned between water (500 mL) and ethyl acetate (1 L × 3). The ethyl acetate–soluble portion (47.0 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 by cyotoxicity against the P388 murine leukemia cells. The active CH_2Cl_2 layer (8.43 g) was subjected to flash chromatography on ODS (5 × 7.5 cm) with MeCN/H_2O (5:5), MeCN/H_2O (7:3), MeCN/H_2O (9:1), MeCN, MeOH, MeOH/$CHCl_3$/H_2O (7:3:0.5), and MeOH/$CHCl_3$/AcOH (6:3:1). Fractions eluted with MeCN/H_2O (7:3) and MeCN/H_2O (9:1) were combined (1.424 g) and successively purified by the following chromatographic systems: (1) Sephadex LH-20 (6 × 88 cm) with MeOH, (2) ODS-MPLC (3 × 100 cm) with MeCN/H_2O (8:2), (3) Sephadex LH-20 (2 × 80 cm) with C_6H_{14}/CH_2Cl_2/MeOH (4:5:1), (4) ODS-MPLC (3 × 100 cm) with MeCN/H_2O (8:2), (5) ODS-HPLC (2 × 50 cm), with MeCN/H_2O (8:2), (6) Sephadex LH-20 (2 × 80 cm) with C_6H_{14}/$CHCl_3$/MeOH (8:1:1), and (6) ODS-HPLC (1 × 25 cm) with MeCN/H_2O (6:4). Ritterazines A (**1**), B (**2**), and C (**3**) (yields, 2.9, 13.4, and 7.8 mg, respectively) were obtained as colorless glassy solids.

2: $[\alpha]_D$ +43.0° (*c* 0.1, MeOH); UV (MeOH) λ_{max} 288 (ε 6880), and 308 (sh) nm; IR (film) 3480, 2960, 2920, 2870, 2850, 1680, 1610, 1460, 1400, 1140, 1120, 1060, 1040, 1000, 980, 940, 880, and 850 cm^{-1}; HR-FABMS (positive) *m/z* 899.5873 ($C_{54}H_{79}O_9N_2$, Δ +8.7 mmu); ^1H and ^{13}C NMR data, see Table 1.

3: $[\alpha]_D$ +72.0° (*c* 0.1, MeOH); UV (MeOH) λ_{max} 285 (ε 8720), and 303 (sh) nm; IR (film) 3400, 2970, 2940, 2880, 1780, 1680, 1610, 1510, 1460, 1380, 1200~1140 (br), 1030, 1000, 980, 950, 870, 800, 720, and 700 cm^{-1}; HR-FABMS (positive) *m/z* 899.5861 ($C_{54}H_{79}O_9N_2$, Δ +7.6 mmu); ^1H and ^{13}C NMR data, see Table 3.

Acetylation of Ritterazine B (2). A solution of ritterazine B (**2**, 20 μg), Ac_2O (0.25 mL), and pyridine (0.5 mL) was stirred at room temperature overnight. The reagents were removed *in vacuo*; the residue was analyzed by FABMS (positive) which showed an $(M + H)^+$ ion at *m/z* 1026, indicating the formation of a triacetate, which was not characterized further.

Preparation of *N*-Methyl Derivatives. Ritterazine B (10.9 mg, 12.1 μmol) was dissolved in methyl iodide (0.9 mL), and the mixture was refluxed for 10 h. Then, additional MeI (0.75 mL) was added to the mixture and refluxing was continued for another 56 h. After cooling, the mixture was freed from the solvent and was purified by ODS–HPLC with MeCN/H_2O/TFA (45:55:0.05) to give *N*3-methylritterazine B (**5**, 2.5 mg yield 21%), *N*3-methylritterazine C (**6**, 1.9 mg yield 16%), *N*2-methylritterazine B (**7**, 2.2 mg yield 18%), and *N*2-methylritterazine C (**8**, 1.3 mg yield 11%).

A mixture of 5 and 6: ^1H NMR (pyridine-d_5) **5** δ 0.72 (3H, s, H19), 0.87 (3H, s, H19′), 1.08 (1H, m, H7α), 1.17 (3H, d, *J* = 7.0 Hz, H21), 1.23 (1H, m, H9′), 1.27 (3H, d, *J* = 7.0, H21′), 1.34 (1H, m, H9), 1.43 (1H, m, H23′β), 1.50 (1H, m, H7β), 1.65 (1H, m, H11β), 1.66 (1H, m, H8), 1.67 (1H, m, H24β), 1.76 (1H, m, H15α), 1.77 (1H, m, H5), 1.77 (1H, m, H15β), 1.79 (1H, m, H5′), 1.80 (1H, m, H6β), 1.86 (1H, m, H24′β), 1.89 (1H, m, H11′β), 2.00 (1H, m, H11α), 2.00 (1H, m, H20), 2.02 (1H, m, H23β), 2.02 (1H, m, H24α), 2.07 (1H, m, H14), 2.10 (1H, m, H23α), 2.13 (1H, m, H24′α), 2.21, (1H, m, H20′), 2.23 (1H, m, H6′α), 2.24 (1H, m, H11′α), 2.41 (1H, m, H8′), 2.50 (1H, ddd, *J* = 14.5, 13.5, 5.0 Hz, H23′α), 2.96 (1H, d, *J* =18.0 Hz, H1′α), 2.96 (1H, m, H4β), 3.04 (1H, m, H4′β), 3.07 (1H, d, *J* =17.0 Hz, H1α), 3.10 (1H, m, H4′α), 3.15 (1H, m, H17), 3.30 (1H, d, *J* =17.0 Hz, H1β), 3.40 (1H, d, *J* =18.4 Hz, H1′β), 3.48 (1H, m, H4α), 3.61 (1H, d, *J* =11.5 Hz, H26′α), 3.64 (1H, m, H12), 4.01 (1H, d, *J* =11.5 Hz, H26′β), 4.04 (1H, m, H7′), 4.17 (1H, m, H12′), 4.55 (3H, s, N3Me), 4.78 (1H, m, H16), 5.22 (1H, br s, H16′), 6.11 (1H, s, H15′); **6** δ 0.72 (3H, s, H19), 0.87 (3H, s, H19′), 1.08 (1H, m, H7α), 1.17 (3H, d, *J* = 7.0 Hz, H21), 1.21 (3H, d, *J* = 7.0 Hz, H21′), 1.23 (1H, m, H9′), 1.34 (1H, m, H9), 1.50 (1H, m, H7β), 1.65 (1H, m, H11β), 1.66 (1H, m, H8), 1.67 (1H, m, H24β), 1.76 (1H, m, H15α), 1.77 (1H, m, H5), 1.77 (1H, m, H15β), 1.79 (1H, m, H5′), 1.80 (1H, m, H6β), 1.89 (1H, m, H11′β), 2.00 (1H, m, H11α), 2.00 (1H, m, H20), 2.02 (1H, m, H23β), 2.02 (1H, m, H24α), 2.07 (1H, m, H14), 2.10 (1H, m, H23α), 2.23 (1H, m, H6′α), 2.24 (1H, m, H11′α), 2.35 (1H, m, H20′), 2.41 (1H, m, H8′), 2.96 (1H, d, *J* =18.0 Hz, H1′α), 2.96 (1H, m, H4β), 3.04 (1H, m, H4′β), 3.07 (1H, d, *J* =17.0 Hz, H1α), 3.10 (1H, m, H4′α), 3.15 (1H, m, H17), 3.30 (1H, d, *J* =17.0 Hz, H1β), 3.40 (1H, d, *J* =18.4 Hz, H1′β), 3.48 (1H, m, H4α), 3.64 (1H, m, H12), 4.04 (1H, m, H7′), 4.14 (1H, m, H12′), 4.55 (3H, s, N3Me), 4.78 (1H, m, H16), 5.24 (1H, br s, H16′), 5.97 (1H, s, H15′).

A mixture of 7 and 8: ^1H NMR (pyridine-d_5) **7** δ 0.75 (3H, s, H19), 0.81 (3H, s, H19′), 1.06 (1H, m, H7α), 1.17 (3H, m, H21), 1.18 (1H, m, H9′), 1.21 (3H, m, H21′), 1.24 (1H, m, H6β), 1.44 (1H, m, H23′β), 1.45 (1H, m, H7β), 1.46 (1H, m, H6α), 1.62 (1H, m, H5), 1.67 (1H, m, H24β), 1.70 (1H, m, H11β), 1.73 (1H, m, H15β), 1.77 (1H, m, H15α), 1.86 (1H, m, H11′β), 1.87 (1H, m, H24′β), 2.00 (1H, m, H20), 2.01 (1H, m, H8), 2.02 (1H, m, H23β), 2.02 (1H, m, H24α), 2.06 (1H, m, H14), 2.07 (1H, m, H5′), 2.10 (1H, m, H23α), 2.15 (1H, m, H11′α), 2.15, (1H, m, H24′α), 2.17 (1H, m, H11α), 2.22 (1H, m, H20′), 2.42 (1H, m, H8′), 2.52 (1H, m, H23′α), 2.87 (1H, d, *J* =17.9 Hz, H1′α), 2.92 (1H, m, H4β), 3.08 (1H, m, H4′β), 3.10 (1H, d, *J* =18.4 Hz, H1α), 3.15 (1H, m, H17), 3.24 (1H, d, *J* =17.9 Hz,

H1′β), 3.27 (1H, m, H4α), 3.45 (1H, d, J =18.4 Hz, H1β), 3.60 (1H, m, H4′α), 3.62 (1H, d, J =11.0 Hz, H26′α), 3.63 (1H, m, H12), 4.00 (1H, d, J =11.0 Hz, H26′β), 4.05 (1H, m, H7′), 4.20 (1H, m, H12′), 4.57 (3H, s, N2Me), 4.76 (1H, m, H16), 5.27 (1H, s, H16′), 6.11 (1H, s, H15′); **8** δ 0.75 (3H, s, H19), 0.81 (3H, s, H19′), 1.06 (1H, m, H7α), 1.17 (3H, m, H21), 1.18 (1H, m, H9′), 1.24 (1H, m, H6β), 1.28 (3H, m, H21′), 1.45 (1H, m, H7β), 1.46 (1H, m, H6α), 1.62 (1H, m, H5), 1.67 (1H, m, H24β), 1.70 (1H, m, H11β), 1.73 (1H, m, H15β), 1.77 (1H, m, H15α), 1.86 (1H, m, H11′β), 2.00 (1H, m, H20), 2.01 (1H, m, H8), 2.02 (1H, m, H23β), 2.02 (1H, m, H24α), 2.06 (1H, m, H14), 2.07 (1H, m, H5′), 2.10 (1H, m, H23α), 2.15 (1H, m, H11′α), 2.17 (1H, m, H11α), 2.35 (1H, m, H20′), 2.42 (1H, m, H8′), 2.87 (1H, d, J =17.9 Hz, H1′α), 2.92 (1H, m, H4β), 3.08 (1H, m, H4′β), 3.10 (1H, d, J =18.4 Hz, H1α), 3.15 (1H, m, H17), 3.24 (1H, d, J =17.9 Hz, H1′β), 3.27 (1H, m, H4α), 3.45 (1H, d, J =18.4 Hz, H1β), 3.60 (1H, m, H4′α), 3.63 (1H, m, H12), 4.05 (1H, m, H7′), 4.20 (1H, m, H12′), 4.57 (3H, s, N2Me), 4.76 (1H, m, H16), 5.27 (1H, s, H16′), 5.98 (1H, s, H15′).

Ritterazine C-7′,26′,12-Tris-(*S*)-(−)-MTPA Ester (9). To a stirred solution of ritterazine C (0.5 mg, 0.56 μmol) in dry pyridine (80 μL) was added (*R*)-(−)-MTPACl (5 mg in 50 μL dry toluene) and the mixture stirred at room temperature. After 24 h additional (*R*)-(−)-MTPACl (5 mg in 50 μL toluene) was added and the mixture stirred for another 6 h at room temperature. The reaction mixture was purified by ODS-HPLC (90% aqueous MeOH → MeOH) to give ritterazine C-7′,26′,12-tris-(*S*)-(−)-MTPA ester (0.6 mg, yield 70%): ^{1}H NMR ($CDCl_3$) δ 0.767 (3H, d, J = 7.3 Hz, H21), 0.809 (3H, s, H19), 0.868 (3H, s, H19′), 0.878 (1H, m, H9), 0.993 (3H, s, H18), 1.002 (3H, s, H18′), 1.020 (3H, d, J = 6.9 Hz, H21′), 1.157 (3H, s, H27′), 1.190 (1H, m, H9′), 1.254 (3H, s, H26), 1.290 (1H, m, H8), 1.342 (3H, s, H27), 1.436 (1H, m, H6′β), 1.527 (1H, m, H11β), 1.621 (1H, m, H11′β), 1.720 (1H, m, H20), 1.764 (1H, m, H15α), 1.814 (1H, m, H15β), 1.880 (1H, m, H5′), 1.940 (1H, m, H20′), 1.960 (1H, m, H11α), 1.970 (1H, m, H11′α), 2.029 (1H, m, H6′α), 2.058 (1H, m, H17), 2.385 (1H, m, H8′), 3.850 (1H, dd, J = 11.3, 4.8 Hz, H12′), 4.229 (1H, d, J = 10.8 Hz, H26′b), 4.406 (1H, d, J = 10.8 Hz, H26′a), 4.543, (1H, dd, J = 7.0, 7.0 Hz, H16), 4.743 (1H, d, J = 1.7 Hz, H16′), 4.890 (1H, dd, J = 9.5, 4.4 Hz, H12), 5.307 (1H, dd, J = 10.6, 10.1 Hz, H7′), 5.460 (1H, dd, J = 1.7, 1.6 Hz, H15′).

Ritterazine C-7′,26′,12-Tris-(*R*)-(+)-MTPA Ester (10). Ritterazine C (0.9 mg, 1.0 μmol) was treated with (*S*)-(+)-MTPACl (5 mg in 50 μL dry toluene) to give ritterazine C-7′,26′,12-tris-(*R*)-(+)-MTPA ester (1.1 mg, yield 84%): ^{1}H NMR ($CDCl_3$) δ 0.784 (3H, s, H18), 0.871 (1H, m, H9), 0.916 (3H, d, J = 7.3 Hz, H21), 0.929 (3H, s, H18′), 1.001 (3H, d, J = 6.9 Hz, H21′), 1.016 (3H, s, H19′), 1.071 (3H, s, H19), 1.171 (3H, s, H27′), 1.190 (1H, m, H9′), 1.254 (3H, s, H26), 1.290 (1H, m, H8), 1.351 (3H, s, H27), 1.406 (1H, m, H11β), 1.615 (1H, m, H6′β), 1.618 (1H, m, H11′β), 1.774 (1H, m, H15α), 1.797 (1H, m, H20), 1.824 (1H, m, H15β), 1.899 (1H, m, H11α), 1.963 (1H, m, H5′), 1.963 (1H, J = 6.9 Hz, H20′), 1.964 (1H, m, H11′α), 2.164 (1H, m, H6′α), 2.284 (1H, m, H17), 2.374 (1H, m, H8′), 3.832 (1H, dd, J = 11.3, 4.8 Hz, H12′), 4.161 (1H, d, J = 10.8 Hz, H26′b), 4.430 (1H, d, J = 10.8 Hz, H26′a), 4.575, (1H, dd, J = 7.0, 7.0 Hz, H16), 4.682 (1H, d, J = 1.7 Hz, H16′), 4.882 (1H, dd, J = 9.5, 4.4 Hz, H12), 5.315 (1H, dd, J = 10.6, 10.1 Hz, H7′), 5.396 (1H, dd, J = 1.7, 1.6 Hz, H15′).

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Supplementary Material Available: Copies of 1D and 2D NMR spectra of **2**, **3**, and **5** (27 pages). This material is contained in libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.

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