Bioactive Marine Metabolites Series. Part 63. Ritterazine A, a Highly Cytotoxic Dimeric Steroidal Alkaloid, from the Tunicate Ritterella tokioka

ARTICLE *in* THE JOURNAL OF ORGANIC CHEMISTRY · OCTOBER 1994

Impact Factor: 4.72 · DOI: 10.1021/jo00100a014

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Ritterazine A, a Highly Cytotoxic Dimeric Steroidal Alkaloid, from the Tunicate *Ritterella tokioka*[1]

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Received July 26, 1994®

Summary: A new cytotoxic dimeric steroidal alkaloid, ritterazine A, has been isolated from the tunicate, *Ritterella tokioka*, and its structure elucidated by extensive spectroscopic analyses.

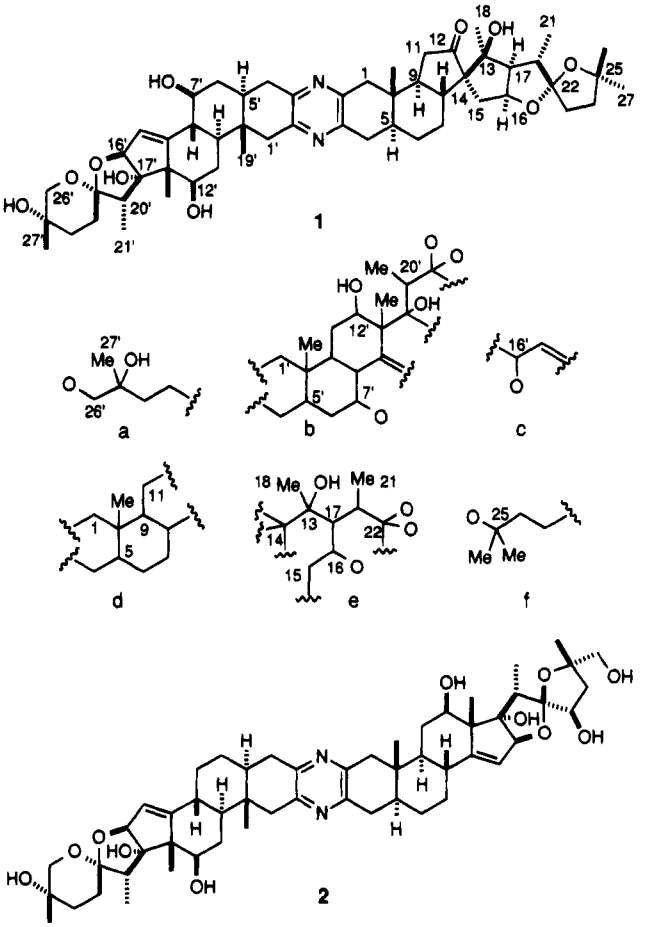
Tunicates have proved to be a rich source of cytotoxic compounds,[2] including such important agents as the didemnins[3] and ecteinascidins.[4] In the course of our search for cytotoxic metabolites from Japanese marine invertebrates the lipophilic extract of the tunicate *Ritterella tokioka* Kott, 1992 (family, Polyclinidae) showed promising activity. Bioassay-directed fractionation afforded ritterazine A which exhibited cytotoxicity against P388 murine leukemia cells with an IC_{50} value of 3.8×10^{-3} μg/mL.

Colonies of the tunicate (5.5 kg)[5] collected off the Izu Peninsula, 100 km southwest of Tokyo, were extracted with ethanol and then acetone. The concentrated extracts were partitioned between water and ethyl acetate; the organic phase was partitioned by a series of solvent systems: *n*-hexane/90% aqueous MeOH and CH_2Cl_2/60% aqueous MeOH. The active CH_2Cl_2 fraction was repeatedly fractionated by ODS and LH-20 chromatographies to afford ritterazine A as a colorless glassy solid (2.9 mg; 5.3×10^{-5} % wet weight).[6]

Ritterazine A[7] (**1**) had a molecular fomula of $C_{54}H_{76}N_2O_{10}$ as established by HR-FABMS. The UV absorption at 287 nm (ϵ 8550) indicated the presence of a pyrazine ring,[8] which was supported by four overlapping ^{13}C NMR signals at δ 145–150.[9]

The ^1H NMR spectrum revealed sharp and well-dispersed signals equivalent to 76 protons, which included seven methyl singlets and two methyl doublets. The ^{13}C NMR spectrum revealed a ketone (δ 221.2), two acetal/hemiacetals (δ 108.0, 119.8), four oxygenated quaternary carbons, four oxygenated methines, and an oxygenated methylene. In addition to pyrazine carbons, two sp^2 carbon signals resonated at δ 121.2 (d) and 152.0 (s).

COSY coupling networks were interrupted by many quaternary carbons and overlapping signals. Therefore, construction of structural units was first carried out by using the well-separated methyl protons in the HMBC spectrum;[10] the resulting units were further extended by analysis of COSY and HOHAHA data, which gave rise to structural units **a**-**f**. Connection of these units and



formation of ether rings were accomplished by HMBC experiments. Connection of units **d** and **e** needs a comment. In the HMBC spectrum the Me-18 signal at δ 1.30 was correlated with carbons at δ 61.7 (C17), 80.9 (C13), and 69.3 (C14), while long-range coupling between these methyl protons and a hydroxyl proton (δ 5.28) was observed in the COSY spectrum; this placed a hydroxyl group at C13. Connectivities between C14 and C15 and between C8 and C14 were implied from HMBC crosspeaks H15α/C13 and H15β/C8, respectively. Location of a ketone (C12) between C11 and C14 was verified by HMBC crosspeaks H11α, H11β, H15α, H15β/C12, and H11α/C14. Connectivities of units **a**–**c** as well as units **e** and **f** were unexceptional, thereby completing two steroid moieties, one with a rearranged skeleton. At this point, we realized that the western hemisphere of the molecule composed of units **a**–**c** was identical with the

® Abstract published in *Advance ACS Abstracts,* October 1, 1994.

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(5) Colonies were cleaned by removing macroepibionts and sands before extraction. There were no concomitant hemichordates.

(6) The dichloromethane phase was chromatographed on ODS with aqueous MeCN. Fractions eluted with MeCN/H_2O (7:3) and MeCN/H_2O (9:1) were combined (1.424 g) and gel-filtered on LH-20 (MeOH) followed by ODS-MPLC (80% aq MeCN), gel-filtration on LH-20 [hexane/CH_2Cl_2/MeOH (4:5:1)], ODS-MPLC (80% aqueous MeCN), ODS-HPLC (80% aqueous MeCN), gel-filtration on LH-20 [hexane/$CHCl_3$/MeOH (8:1:1)], and ODS-HPLC (60% aqueous MeCN).

0022-3263/94/1959-6164$04.50/0

Table 1. NOE and H–C Long-Range Coupling by HMBC Spectrum of Ritterazine A

left side				right side			
no.	δ	NOE	HMBC	no.	δ	NOE	HMBC
H-1′α	2.70	H9′	C2′, C9′, C10′, C19′	H-1α	2.76	H9	C2, C9, C10, C19
H-1′β	3.17	H11′α	C2′, C3′, C5′, C10′, C19	H-1β	2.91	H11α, H11β	C2, C5, C10, C19
H-4′α	3.02	H6′α	C3′, C5′, C10′	H-4α	2.97	H6α	C3, C5, C10
H-4′β	2.81	H6′β	C3′, C5′	H-4β	2.69	H6β	C3, C5, C10
H-5′	1.88	H9′, H7′	C19′	H-5	1.74	H7α	C10
H-6′α	2.22	H4′α, H7′	C8′	H-6α	1.65	H4α, H7β	
H-6′β	1.77	H4′β	C7′, C8′	H-6β	1.45	H4β, H7β	
H-7′	4.07	H5′, H6′α, H15′		H-7α	1.36	H5, H15β	
				H-7β	2.27	H6α, H6β, 13OH	
H-8′	2.43	H19′	C7′, C9′, C14′, C15′	H-8	2.40	13OH, H19	C14
H-9′	1.19	H1′α, H5′		H-9	1.62	H1α, H15α, H15β	C1, C7, C10, C11, C19
H-11′α	2.17	H1′β, H12′	C9′, C12′, C13′	H-11α	2.35	H1β	C8, C12, C14
H-11′β	1.87	H18′	C9′, C12′, C13′	H-11β	2.14	H1β	C9, C10, C12
H-12′	4.22	H11′α, H16′	C17′, C18′				
12′-OH	4.66		C11′, C12′				
				13-OH	5.28	H7β, H8, H15β, H20	C13, C17, C16, C18
H-15′	6.13	H7′	C8′, C13′, C14′, C16′, C17′	H-15α	2.08	H9, H16	C12, C13, C17
				H-15β	2.24	H7α, H9, 13OH	C8, C12, C14, C16
H-16′	5.25	H12′, H26′β	C14′, C17′	H-16	5.20	H15α	C13, C22
17′OH	5.00		C13′, C17′	H-17	2.85	H21	C15, C16, C18, C20, C21, C22
H-18′	1.34	H11′β, H20′	C12′, C13′, C14′, C17′	H-18	1.30		C13, C14, C17
H-19′	0.88	H8′	C1′, C5′, C9′, C10′	H-19	0.81	H8	C1, C5, C9, C10
H-20′	2.22	H18′, H23′α, H23′β	C13′, C17′, C21′, C22′, C23′	H-20	2.54	13OH	C13, C16, C17, C21, C22
H-21′	1.27		C17′, C20′, C22′	H-21	0.98	H17	C17, C20, C22
H-23′α	2.50	H20′	C22′, C24′	H-23α	1.94		C24
H-23′β	1.45	H20′, H24′α	C22′, C25′	H-23β	1.90		C22, C25
H-24′α	1.86	H23′β, H24′β		H-24α	2.01	H24β, H27	C23, C25, C26, C27
H-24′β	2.15	H24′α	C23′	H-24β	1.68	H24α	C22
25′OH	3.64		C26′				
H-26′α	3.61	H26′β	C22′, C24′, C25′	H-26	1.12		C24, C25, C27
H-26′β	4.01	H16′, H26′α, H27	C25′				
H-27′	1.23	H26′β	C24′, C25′, C26′	H-27	1.42	H24α	C24, C25, C26

corresponding portion of cephalostatin 7 (**2**),[11] a cytotoxic metabolite of the hemichordate *Cephalodiscus gilchristi*, except for the C7′ substituent. With this in mind, we could connect the two steroid units through a tetrasubstituted pyrazine ring,[12] which satisfied the molecular formula. This structure was also in good agreement with our spectral data. However, the orientation of the two steroid units could not be determined on the basis of NMR data. The structure was drawn as in the case of cephalostatin 1, which had been secured by X-ray diffraction.[13]

Relative stereochemistry of each steroid unit was deduced by ^{1}H, ^{1}H-coupling constants and NOESY data. H5, H8, and H9 were assigned as axial on the basis of large vicinal coupling constants. An intense NOESY cross peak between CH_3-19 and H8 indicated that CH_3-18 was axial. 13-OH revealed NOESY cross peaks with H8, H7β, and H20, allowing us to deduce the stereochemistry of ring D and C20. Stereochemistry of ring E was established on the basis of NOESY cross peaks CH_3-21/H17, H16/H15α, H15α/H9, and H15β/H7α. ^{1}H NMR data for the western hemisphere except H7′ were almost superimposable on those of cephalostatin 7, thereby suggesting that the stereochemistry of the western hemisphere was identical with that of cephalostatin 7. H7′ was assigned as axial on the basis of coupling constant analysis. Relative stereochemistry of the western hemisphere was further substantiated by NOESY data. The absolute stereochemistry of the western

(7) **1**: $[\alpha]_D$ +112.0° (*c* 0.1, MeOH); UV (MeOH) λ_{max} 287 (ε 8580) and 308 sh nm; IR (film) 3420, 2970, 2940, 2880, 2360, 1770, 1730, 1600, 1450, 1400, 1260, 1230, 1160, 1130, 1080, 1040, 990, 970, 960, 940, 890, 870, and 820 cm^{-1}; HR-FABMS (positive; glycerol matrix) *m/z* 913.5576 and 895.5408, corresponding to the molecular formulas of $C_{54}H_{77}N_2O_{10}$, (Δ −0.2 mmu) and $C_{54}H_{75}N_2O_9$, (Δ −6.5 mmu), respectively; ^{13}C-NMR data in pyridine-d_5 at 303 K 8.1 (C21′), 10.9 (C19), 11.8 (C19′), 12.6 (C18′), 19.0 (C21), 23.5 (C18), 27.0 (C27′), 27.5 (C23′), 28.6 (C26), 29.1 (C6), 29.2 (C11′), 30.4 (C27), 30.6 (C7), 32.7 (C23), 33.2 (C24′), 35.6 (C4′ and 10), 35.9 (C4, 10′ and 15), 37.4 (C24), 38.4 (C6′), 40.0 (C5′), 40.6 (C8), 40.8 (C20), 40.9 (C11), 41.9 (C5), 42.9 (C8′), 45.9 (C1′), 46.7 (C1), 48.3 (C20′), 50.1 (C9), 51.2 (C9′), 56.1 (C13′), 61.7 (C17), 65.8 (C25′), 69.3 (C14), 69.4 (C7′), 70.2 (C26′), 75.8 (C12′), 80.9 (C13), 82.7 (C25), 82.9 (C16), 93.3 (C17′), 94.0 (C16′), 108.0 (C22′), 119.8 (C22), 121.2 (C15′), 148.7 (C3′), 149.0 (C2), 149.2 (C2′ and C3), 152.0 (C14′), 221.2 (C12); ^{1}H-NMR data in pyridine-d_5 at 303 K 0.81 (3H, s, H19), 0.88 (3H, s, H19′), 0.98 (3H, d, *J* = 7.3 Hz, H21), 1.12 (3H, s, H26), 1.19 (1H, ddd, *J* = 12.2, 10.9, 4.0 Hz, H9′), 1.23 (3H, s, H27′), 1.27 (3H, d, *J* = 6.9 Hz, H21′), 1.30 (3H, s, H18), 1.34 (3H, s, H18′), 1.36 (1H, m, H7α), 1.42 (3H, s, H27), 1.45 (1H, m, H6β), 1.45 (1H, m, H23′β), 1.62 (1H, m, H9), 1.65 (1H, m, H6α), 1.68 (1H, m, H24β), 1.74 (1H, m, H5), 1.77 (1H, dd, *J* = 12.7, 10.3 Hz, H6′β), 1.86 (1H, m, H24′α), 1.87 (1H, m, H11′β), 1.88 (1H, m, H5′), 1.90 (1H, m, H23β), 1.94 (1H, m, H23α), 2.01 (1H, dd, *J* = 11.3, 3.5 Hz, H24α), 2.08 (1H, m, H15α) 2.14 (1H, m, H11β), 2.15 (1H, m, H24′β), 2.17 (1H, m, H11′α), 2.22 (1H, m, H6′α), 2.22 (1H, q, *J* = 6.9 Hz, H20′), 2.24 (1H, m, H15β), 2.27 (1H, m, H7β), 2.35 (1H, dd, *J* = 16.5, 6.3 Hz, H11α), 2.40 (1H, ddd, *J* = 12.1, 11.9, 3.6 Hz, H8), 2.43 (1H, dd, *J* = 10.9, 10.6 Hz, H8′), 2.50 (1H, ddd, *J* = 13.3, 13.1, 4.9 Hz, H23′α), 2.54 (1H, dq, *J* = 1.5, 7.3 Hz, H20), 2.69 (1H, dd, *J* = 17.8, 12.7 Hz, H4β), 2.70 (1H, d, *J* = 16.8 Hz, H1′α), 2.76 (1H, d, *J* = 16.4 Hz, H1α), 2.81 (1H, dd, *J* = 17.7, 13.0 Hz, H4′β), 2.85 (1H, dd, *J* = 9.3, 1.5 Hz, H17), 2.91 (1H, d, *J* = 16.4 Hz, H1β), 2.97 (1H, dd, *J* = 17.8, 5.5 Hz, H4α), 3.02 (1H, dd, *J* = 17.7, 5.1 Hz, H4′α), 3.17 (1H, d, *J* = 16.8 Hz, H1′β), 3.61 (1H, dd, *J* = 11.5, 2.6 Hz, H26′α), 3.64 (1H, br s, 25′OH), 4.01 (1H, d, *J* = 11.5 Hz, H26′β), 4.07 (1H, ddd, *J* = 10.6, 9.8, 5.6 Hz, H7′), 4.22 (1H, ddd, *J* = 11.3, 4.8, 1.7 Hz, H12′), 4.66 (1H, br s, 12′OH), 4.89 (1H, br s, 17′OH), 5.20 (1H, ddd, *J* = 9.3, 3.9, 2.9 Hz, H16), 5.25 (1H, d, *J* = 1.9 Hz, H16′), 5.28 (1H, br s, 13OH), 6.02 (1H, br s, 7′OH), 6.13 (1H, dd, *J* = 1.9, 1.8 Hz, H15′).

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(12) Assignments for C2, C3, C2′, and C3′ may be interchanged.

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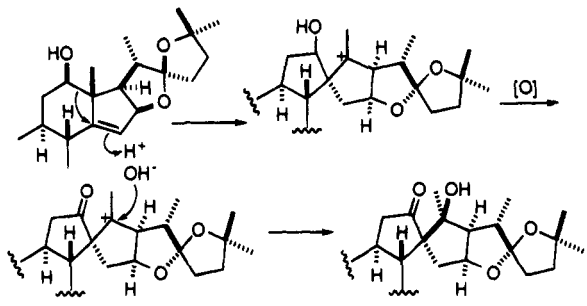
hemisphere of ritterazine A may be the same as for the well-established steroid nucleus.[14]

Scheme 1. Proposed Biosynthetic Pathway of Ritterazine A(1)



The biogenesis of the eastern hemisphere of the molecule is derivable from a 12-hydroxy-Δ^{14}-steroid skeleton as shown in Scheme 1. Isolation of closely related dimeric alkaloids from two different phyla poses a problem of the true origin.

(14) Ritterazine A was very sensitive to acid. Attempts at conversion to the ritterazine A–MTPA ester were unsuccessful.

Acknowledgment. We are grateful to Professor P. J. Scheuer of The University of Hawaii for reading the manuscript. Thanks are also due to Dr. T. Nishikawa of Nagoya University for identification of the tunicate, to Drs. K. Yazawa, K. Yamada, and K. Kinoshita of the Sagami Chemical Research Center for their help with the cytotoxicity assay, and to Dr. Y. Nakao of this laboratory for measuring HR-FABMS. This work was partly supported by a Grant-in-Aid for Science Research from the Ministry of Education, Science, and Culture of Japan.

Supplementary Material Available: Copies of 1D and 2D NMR spectra of **1** (15 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.