Cinachyrolide A: A Potent Cytotoxic Macrolide Possessing Two Spiro Ketals from Marine Sponge *Cinachyra* sp.[1]

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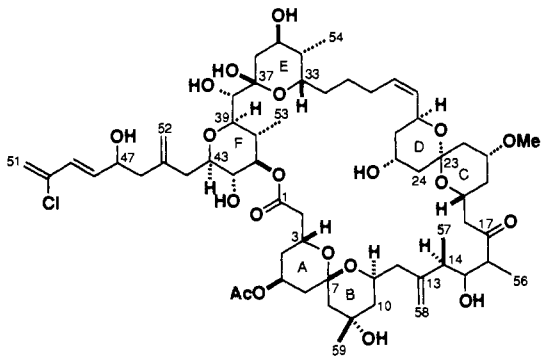
Abstract: A highly cytotoxic macrolide, cinachyrolide A, has been isolated from marine sponge *Cinachyra* sp. The gross structure was determined mainly by extensive 2D NMR experiments. The relative stereochemistry of the six oxane rings is proposed on the basis of NOESY data.

Cytotoxic and/or antitumor macrolides have been encountered in marine sponges, e.g., tedanolide,[2] 13-deoxytedanolide,[3] halichondrins,[4] swinholides,[5] or bistheonellides.[6] In the course of our search for bioactive metabolites from marine invertebrates,[1] we collected a sponge of the genus *Cinachyra*,[7] which proved to be highly cytotoxic during initial screening. Bioassay-guided isolation afforded a new cytotoxic macrolide, cinachyrolide A (**1**). The isolation and structure elucidation of this compound are described in this report.



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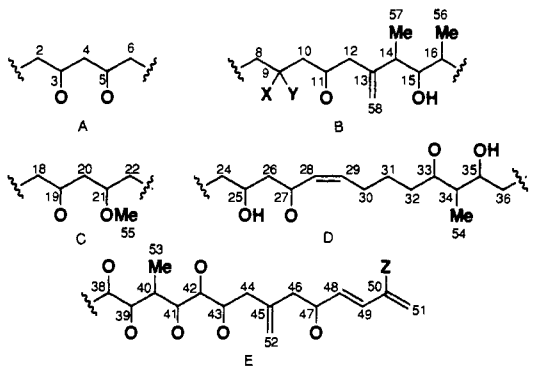
The ether-soluble material of the EtOH extract of the sponge (6.6 kg wet weight) was partitioned between 90% MeOH and *n*-hexane. The aqueous MeOH layer was fractionated by ODS flash chromatography. A fraction eluted with 70% MeOH was repeatedly purified by ODS HPLC to yield cinachyrolide A as a colorless solid (1.1 mg), which was highly cytotoxic against L1210 murine leukemia cells with an IC_{50} of <0.6 ng/mL.

Cinachyrolide A had a molecular formula of $C_{61}H_{93}ClO_{20}$, established by HRFABMS (m/z 1203.5897 $(M + Na)^+$, Δ5.1 mmu). The UV spectrum indicated the presence of a conjugated diene, which was supported by NMR data. Fortunately, the ^1H NMR spectra measured in CD_3OD and in DMSO-d_6 displayed well-separated signals, which enabled us to determine the gross structure for **1**. Interpretation of the COSY spectrum together with the HOHAHA (Figure 1)[9] and HMQC[10] spectra led to structural units A–E.[11]



Unit A. In the COSY spectrum, C2 methylene protons (δ 2.60, 2.62), which were indicated to be vicinal to a carbonyl carbon as judged by the chemical shifts, were correlated to an oxygenated methine at δ 4.39 (H3), which was in turn correlated to methylene protons at δ 1.60 and 1.76 (H_24). These methylene protons were also coupled to a low-field oxygenated methine at δ 5.03 (H5), which was further coupled to other methylene protons at δ 1.73 and 1.92 (H_26). The chemical shift of H5 suggested that C5 was participating in an ester linkage; C6 was adjacent to an sp^3 quaternary carbon.

Unit B. Although the C8 methylene was flanked by two sp^3 quaternary carbons, one of the methylene protons at δ 1.67 (H8b) exhibited a *W*-type coupling with H10b (δ 1.55). Connectivities from C10 to C12 were readily inferred from the COSY spectrum. The chemical shift of δ 4.64 ppm indicated that C11 was oxygenated, while the shifts for H_212 (δ 2.21, 2.26) implied that C12 was juxtaposed on an sp^2 carbon. Allylic couplings, H12a/H58b, H12b/H58b, and H14/H58a, allowed connection to C12 and C14 through C13. Connectivities from C14 to C16 were

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(7) The globular sponge was collected by scuba diving (–5 to –15 m) off Hachijo-jima Island, 300 km south of Tokyo, and was identified as *Cinachyra* sp. (Tetillidae) by Professor P. R. Bergquist. Only a few reports on metabolites from sponges of the family Tetillidae are found in the literature.[8]

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(11) For the assignment of nonequivalent methylene protons, higher field methylene protons were suffixed by a (e.g., Ha), while lower field methylene protons were suffixed by b (e.g., Hb).

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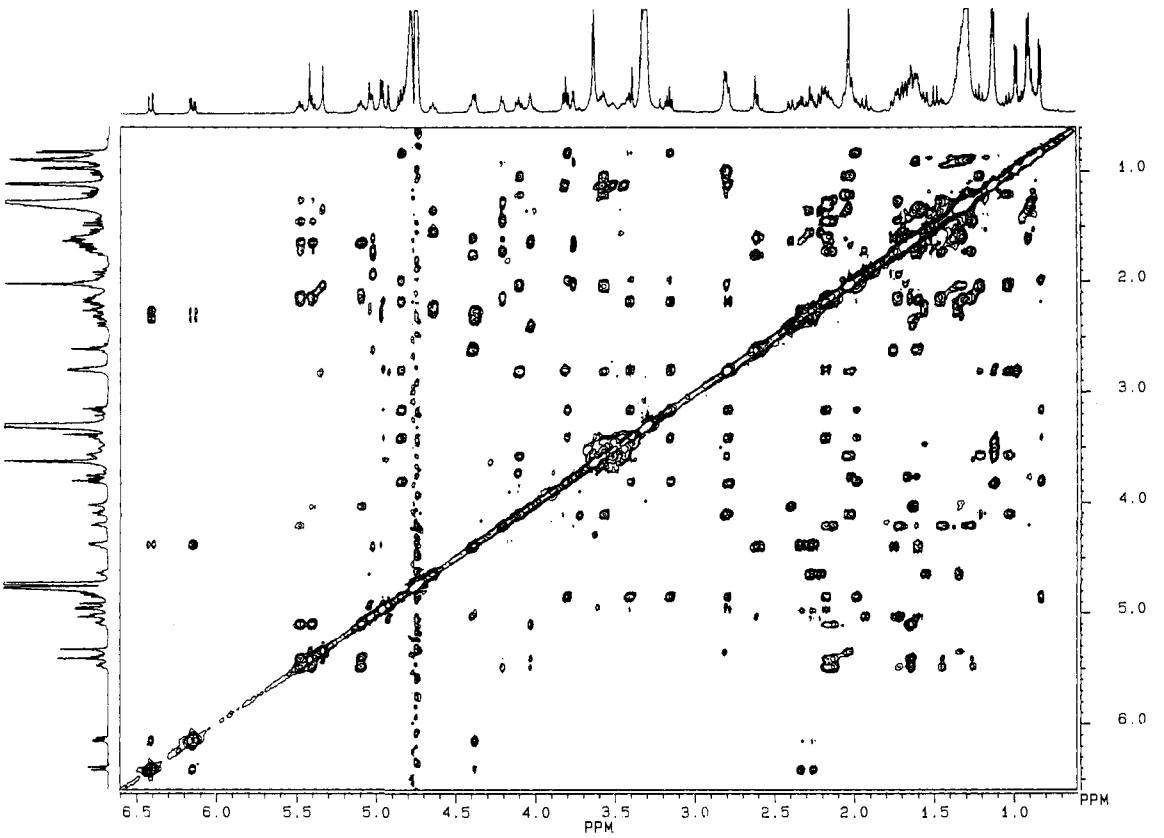

Figure 1. HOHAHA spectrum of cinachyrolide A in CD_3OD.

straightforward. Although H14 (δ 2.82) and H16 (δ 2.80) resonated close to each other, the chemical shift difference was large enough to distinguish between their COSY cross-peaks.

Unit C. Geminally coupled low-field methylene protons at δ 2.84 and 2.87 ($H_2$18) were correlated with H19, which was further coupled to highly nonequivalent methylene protons at δ 1.03 and 2.01 ($H_2$20). Another oxygenated methine at δ 3.57 showed cross-peaks with $H_2$20 and $H_2$22.

Unit D. Since three methylene protons (H24a, H26a, and H26b) resonated at δ 1.63–1.65 in CD_3OD, connectivities from C24 to C27 could not be traced from the COSY spectrum. This problem was overcome by measuring the sample's spectrum in DMSO-d_6, which gave better dispersion of the relevant signals. An oxygenated methine at δ 5.10 was correlated with $H_2$26 and H28; the latter was a part of a *Z* olefin (δ 5.40, $J_{28,29}$ = 10.3 Hz) coupled with H29 at δ 5.48. H29 was further correlated with allylic methylene protons at δ 2.13 and 2.18 ($H_2$30), which were in turn coupled to other methylene protons at δ 1.58 and 1.70 ($H_2$31). $H_2$31 were again correlated with high-field methylene protons at δ 1.28 and 1.44 ($H_2$32), which showed a cross-peak with an oxygenated methine at δ 4.21 (H33). Similarly, connectivities from C33 to C36 were established by the COSY spectrum.

Unit E. A left-half portion of unit E was highly oxygenated. Connectivities from C38 to C44 were evident from COSY cross-peaks, viz., H38/H39, H39/H40, H40/53-Me, H40/H41, H41/H42, H42/H43, H43/H44a, and H43/H44b. C44 and C46 were connected via C45 on the basis of allylic couplings observed in the COSY spectrum: H44a/H52a, H44b/H52a, H44b/H52b, and H46b/H52b. Methylene protons at δ 2.26 and 2.33 ($H_2$46) were coupled to an oxygenated methine at δ 4.34 (H47), which was further correlated to *E* olefinic protons ($J_{48,49}$ = 15.8 Hz) at δ 6.14 (H48) and 6.41 (H49). Incidentally, H48 and H49 displayed long-range couplings with the terminal methylene protons at δ 4.92 and 5.04 ($H_2$51).

Large vicinal coupling constants ($J_{10a,11}$ = 11.2 Hz, $J_{19,20a}$ = 11.4 Hz, and $J_{21,22a}$ = 12.1 Hz) as well as *W*-type couplings ($J_{4b,6b}$ = 1.6 Hz, $J_{8b,10b}$ = 2.4 Hz) suggested the presence of six-membered rings. Cross-peaks between hydroxyl and carbinol methine protons in the COSY spectrum measured in DMSO-d_6 led to the location of four secondary hydroxyl groups (C15-OH, C25-OH, C35-OH, and C47-OH).

Assembly of the Partial Structural Units. Connectivities of units A–E via nonprotonated carbons were established on the basis of HMBC[12] and NOESY[13] data. Though the limited sample made it impossible to measure the ^{13}C NMR spectrum for **1**, chemical shifts and multiplicities of carbon signals (Table I) could be obtained from the HMQC and HMBC spectra (Figure 2).[14] In addition to units A–E, there were signals assignable to a ketone, two ester carbonyls, three acetal/hemiacetals, a quaternary oxygenated carbon, an *O*-methyl, and an acetyl. Placement of an ester on C2, connectivities from C6 to C8 via a C7 ketal, and linkage of C16 to the C17 ketone were based on HMBC cross-peaks; H2/C1, H6b/C7, H8a/C7, and 16-Me/C17. A singlet of methyl protons at δ 1.13 (56-Me) was correlated with C8, C10, and a singlet carbon at δ 69.8 (C9) in the HMBC spectrum, while these methyl protons exhibited a COSY cross-peak with a hydroxyl proton at δ 3.98 in DMSO-d_6. Therefore, X and Y on C9 in unit B must be a methyl and a hydroxyl. The ^1H NMR signal of one of the methylene protons on C18 disappeared during storage in CD_3OD due to exchange with a deuterium, thereby revealing that it was vicinal to the ketone. An *O*-methyl group on C21 was corroborated by an HMBC cross-peak: OCH_3/C21. Although the C23 ketal showed an HMBC cross-peak only with

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(14) Multiplicities of proton signals were determined from the resolution-enhanced ^1H NMR spectrum as well as the ECOSY spectrum (Griesinger, C.; Sørensen, O. W.; Ernst, R. R. *J. Magn. Reson.* **1987**, *75*, 474–492).

Table I. ^1H and ^{13}C NMR Data for Cinachyrolide A in CD_3OD

no.	^1H, ppm (mult, J(Hz))	^{13}C, ppm	HMBC	no.	^1H, ppm (mult, J(Hz))	^{13}C, ppm	HMBC
1		173.5		30a	2.13 (m)	28.0	
2a	2.60 (dd, 9.6, 12.5)	39.8	C1, C3	30b	2.18 (m)		
2b	2.62 (dd, 12.5, 2.9)		C1, C3	31a	1.58 (m)	27.5	
3	4.39 (m)	62.7		31b	1.70 (m)		
4a	1.60 (m)	34.8		32a	1.28 (m)	33.0	
4b	1.76 (m)			32b	1.44 (m)		
5	5.03 (m)	67.8	C6	33	4.21 (ddd, 9.9, 2.3, 2.3)	67.7	
6a	1.73 (d, 15.2)	38.3		34	1.61 (qdd, 7.2, 3.1, 2.3)	39.8	
6b	1.92 (dd, 15.2, 1.6)		C4, C5, C7	35	3.76 (q, 3.1)	72.0	
7		99.5		36a	1.65 (dd, 15.5, 3.1)	34.0	
8a	1.48 (d, 14.4)	46.6	C7	36b	2.05 (dd, 15.5, 3.1)		
8b	1.67 (dd, 14.4, 2.4)		C7, C9, C10	37		98.8	
9		69.8		38	3.39 (d, 1.3)	73.3	C37
10a	1.33 (dd, 13.9, 11.2)	45.1	C12	39	3.80 (dd, 10.0, 1.3)	81.7	C41
10b	1.55 (dt, 13.9, 2.4)		C9	40	1.98 (qdd, 6.8, 11.0, 10.0)	37.6	
11	4.64 (ddd, 11.2, 9.5, 2.4)	64.9		41	4.84 (dd, 11.0, 9.3)	80.5	C1
12a	2.21 (brd, 13.5)	44.3	C13, C58	42	3.15 (dd, 9.3, 9.3)	73.6	C41, C43, C44
12b	2.26 (dd, 13.5, 9.5)		C11, C13, C14, C58	43	3.41 (dd, 10.1, 9.3)	79.8	C42
13		148.5		44a	2.17 (dd, 16.8, 10.1)	40.5	C43, C45, C46, C52
14	2.82 (qd, 7.0, 1.6)	37.6	C13, C57, C58	44b	2.79 (d, 16.8)		C45, C52
15	3.81 (dd, 10.2, 1.6)	73.7	C13, C16, C57	45		144.0	
16	2.80 (qd, 10.2, 6.9)	49.9	C15, C17	46a	2.26 (dd, 13.5, 6.3)	44.3	C44, C45, C47, C48, C52
17		215.0		46b	2.33 (dd, 13.9, 7.4)		C45, C47, C48, C52
18a	2.84 (dd, 18.0, 9.8)	51.8	C19	47	4.37 (ddd, 7.4, 6.3, 5.9)	70.9	C45, C46, C48, C49
18b	2.87 (dd, 18.0, 1.6)			48	6.14 (dd, 15.8, 5.9)	139.1	C47, C50
19	4.11 (m)	66.7		49	6.41 (d, 15.8)	127.8	C47, C50, C51
20a	1.03 (q, 11.4)	38.1	C19, C21	50		139.9	
20b	2.01 (m)			51a	5.33 (s)	116.1	C49, C50
21	3.57 (m)	74.6		51b	5.41 (s)		C49, C50
22a	1.20 (t, 12.1)	43.8	C21, C23, C24	52a	4.96 (s)	116.2	C44, C46
22b	2.06 (m)			52b	4.97 (s)		C44, C46
23		99.6		53	0.83 (d, 6.7)	12.7	C39, C40, C41
24a	1.63 (dd, 15.4, 4.0)	34.7		54	0.90 (d, 7.2)	11.6	C33, C34, C35
24b	2.40 (brd, 15.4)			55	3.33 (s)	55.7	C21
25	4.03 (m)	65.1		56	1.12 (d, 6.9)	17.5	C15, C16, C17
26	1.65 (m)	38.9		57	0.98 (d, 7.0)	11.1	C13, C14, C15
	1.65 (m)			58a	4.92 (s)	114.8	C12, C14
27	5.10 (dt, 4.6, 9.7)	61.7		58b	5.04 (s)		C12, C14
28	5.40 (dd, 10.3, 9.7)	131.6		59	1.13 (s)	14.0	C8, C9, C10
29	5.48 (dt, 5.0, 10.3)	134.1		60		172.5	
				61	2.03 (s)	21.3	C60

H22a, NOESY cross-peaks (H19/H24b and H21/H24b) as well as chemical shift values for $H_2$24 (1.63 and 2.40 ppm) and C24 (34.7 ppm) indicated that C24 was adjacent to C23. The last ketal/hemiketal carbon at δ 98.8 (C37) was correlated with H38. This proton showed a COSY cross-peak with an exchangeable proton (37-OH) which was in turn coupled to H36a, thereby placing the C37 hemiketal carbon between C36 and C38. The chemical shift for C50 (139.9 ppm) indicated that it had a chloro substituent.

Location of acetal and ether linkages was determined by the NOESY data. A cross-peak between H3 and H11 indicated that oxygens on C3 and C11 were forming a spiro ketal with C7 (rings A and B). Multiple correlations between H19/H24b and H21/H24b allowed placement of the second spiro ketal (rings C and D) between C23 and the oxygens on C19 and C27. An hemiacetal (ring E) was located between C33 and C37 on the basis of a cross-peak between 37-OH and H33, whereas an ether linkage (ring F) between C39 and C43 was secured by cross-peak H39/H43. Finally, a lactone was evident between C1 and the oxygen on C41 on the basis of an HMBC correlation of H41/C1, which was weak but distinguishable from noise. The chemical shift for H5 (δ 5.03) placed the remaining acetoxyl group on C5 to complete the gross structure.

Stereochemistry. The relative stereochemistry of the six oxane rings was established by ^1H NMR coupling constants and NOESY data (Chart I).

(1) C1–C14 Portion (I). H3 was assigned as axial on the basis of a large coupling with H4a, while H5 was assigned as equatorial because it did not exhibit any large coupling with adjacent axial protons. NOESY cross-peaks 59-Me/H8a, 59-Me/H8b, and 9-OH/H11 revealed that 59-Me was equatorial and that the hydroxyl group on C9 was axial. The axial nature of H11 was evident from a 11.2 Hz coupling with H10a. NOESY cross-peaks H3/H11 and H6b/H8a were crucial in the assignment of stereochemistry at C7, allowing it to form a thermodynamically stable spiro ketal with both ketal oxygens in axial positions.[15] Further, NOESY cross-peaks H58a/OAc, H58b/OAc, H14/H11, and 57-Me/H3 placed a constraint on the C12–C14 portion, allowing us to correlate the relative stereochemistry at C14 with the stereochemistry of rings A and B.

(2) C18–C30 Portion (II). H19 and H21 were assigned as axial from the fact that both protons were coupled to H20a by 11.4 Hz, whereas H25 was assigned as equatorial on the basis of small coupling constants with the methylene protons on C24 and C26. H27 was coupled to $H_2$26 by 4.6 and 9.7 Hz, thereby assigning H27 to be axial. NOESY cross-peaks H19/H24b and H22b/H24a allowed us to establish the stereochemistry at C23: the ketal oxygen in ring C was axial while ketal oxygen in ring D was equatorial, violating the anomeric effect. If the spiro ketal were formed with two axial oxygens, the C27 substituent should have become axial; therefore, placement of the substituent on C27 at the equatorial position took precedence over the anomeric effect. ROESY cross-peaks H27/H30a and H27/H30b were also observed.

(3) C33–C43 Portion (III). The axial disposition of H33 and 54-Me was substantiated by NOESY cross-peaks, H33/37-OH

(15) Deslongchamps, P. *Stereoelectronic Effects in Organic Chemistry*; Pergamon Press: Oxford, 1983.

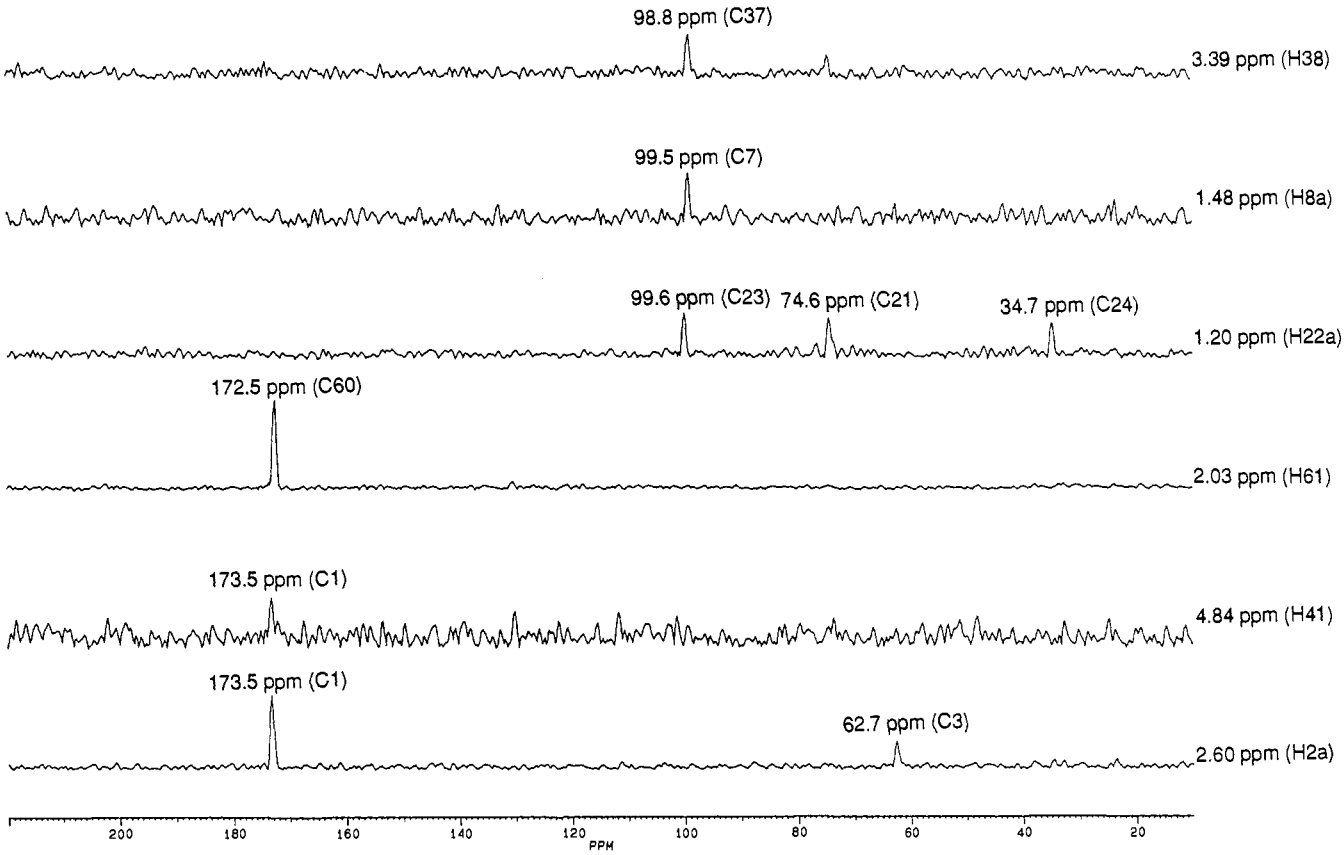
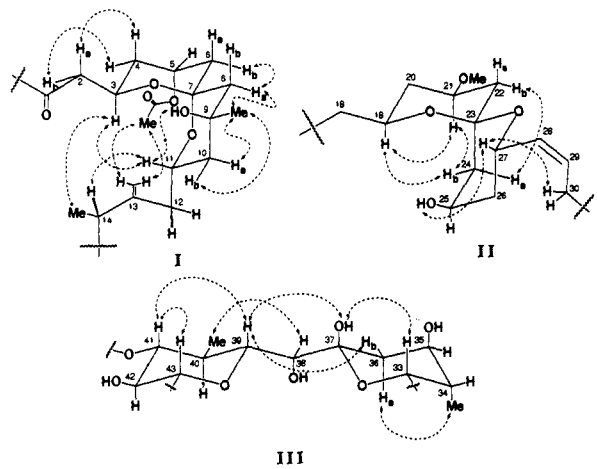


Figure 2. Slices from the HMBC spectrum in CD_3OD indicating correlations with nonprotonated carbons.

Chart I



and 54-Me/H36a, while the equatorial orientation of H35 was deduced from a coupling constant of 3.1 Hz with each of H34, H36a, and H36b. H39, H40, H41, H42, and H43 were all axial on the basis of coupling constants ($J_{39,40}$ = 10.0 Hz, $J_{40,41}$ = 11.0 Hz, $J_{41,42}$ = 9.3 Hz, $J_{42,43}$ = 9.3 Hz). A coupling constant of 1.3 Hz between H38 and H39 as well as NOESY cross-peaks H36a/H38, 37-OH/H39, and H38/53-Me put rotational constraints on the C37–C38 and C38–C39 bonds, allowing us to interrelate the relative stereochemistries of ring E, C38, and ring F.

Conclusion. Cinachyrolide A is the first member of an unprecedented class of natural products, although a number of macrolide antibiotics possessing one spiro ketal, e.g., avermectins,[16] oligomycins, cytovaricin, and rutamycin, have been known from actinomycetes.[17] The remarkable cytotoxicity of cinachyrolide A may provide a useful model for anticancer drugs.

Experimental Section

General Procedures. Optical rotation was determined with a JASCO DIP-371 digital polarimeter. Mass spectra were measured on a JEOL SX 102 mass spectrometer. The ultraviolet spectrum was recorded on a Hitachi 330 spectrophotometer.

Isolation. The frozen sponge (6.6 kg wet weight) was extracted in a blender with EtOH (3 × 20 L). The residue was further extracted with acetone. The combined extracts were evaporated and partitioned between Et_2O and H_2O. The lipophilic portion was further partitioned between 90% MeOH and *n*-hexane. The aqueous MeOH layer was subjected to ODS flash chromatography with increasing amounts of MeOH in water. A fraction eluted with 70% MeOH was fractionated by ODS HPLC on a CAPCELL PAK C_{18} AG120-5 column using a gradient elution from 50% to 80% MeOH. The fraction showing cytotoxicity was purified by ODS HPLC on a COSMOSIL $5C_{18}$-AR column with 40% MeCN. Further purification of **1** by ODS HPLC on a COSMOSIL $5C_{18}$-AR column with 70% MeOH yielded 1.1 mg of **1** as a colorless solid: $[\alpha]^{23}_D$ +5.4° (*c* 0.05, MeOH); UV (MeOH) λ_{max} 230 nm (ϵ 8500); HRFABMS *m/z* 1203.5897 $(M + Na)^+$, $(C_{61}H_{93}{}^{35}ClO_{20}Na)$; ^1H NMR (DMSO-d_6, 600 MHz) 6.40 (d, *J* = 15.2 Hz, H49), 6.06 (dd, *J* = 15.2, 5.3 Hz, H48), 5.54 (s, H51b), 5.37 (s, H51a), 5.36 (m, H29), 5.35 (m, H28), 5.32 (m, 38-OH), 5.32 (m, 42-OH), 4.99 (m, 47-OH), 4.95 (brt, *J* = 10.8 Hz, H27), 4.89 (m, H5), 4.89 (s, H58b), 4.84 (s, H52b), 4.80 (s, H52a), 4.79 (s, H58a), 4.75 (dd, *J* = 8.5, 1.9 Hz, 25-OH), 4.71 (s, 37-OH), 4.66 (dd, *J* = 10.8, 9.2 Hz, H41), 4.61 (brt, *J* = 11.0 Hz, H11), 4.23 (m, H47), 4.20 (brt, *J* = 12.1 Hz, H3), 4.15 (m, 15-OH), 4.11 (d, *J* = 6.7 Hz, 35-OH), 4.03 (brd, *J* = 10.3 Hz, H33), 3.98 (s, 9-OH), 3.95 (brt, *J* = 11.9 Hz, H19), 3.86 (m, H25), 3.64 (d, *J* = 9.8 Hz, H39), 3.60 (m, H15), 3.57 (m, H35), 3.51 (m, H21), 3.35 (m, H43), 3.25 (s, H38), 3.20 (s, H55), 3.03 (dd, *J* = 9.2, 8.9 Hz, H42), 2.71 (m, H2b), 2.71 (d, *J* = 16.9 Hz, H44b), 2.64 (m, H16), 2.61 (m, H14), 2.61 (m, H18), 2.51 (dd, *J* = 11.4, 18.5 Hz, H2a), 2.29 (brd, *J* = 15.6 Hz, H24b), 2.22 (dd, *J* = 14.6, 7.2 Hz, H46b), 2.12 (m, H12b), 2.12 (m, H12a), 2.10 (m, H46a),

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2.04 (m, H30b), 2.04 (m, H30a), 2.00 (m, H44a), 1.96 (s, OAc), 1.95 (m, H20b), 1.95 (m, H22b), 1.83 (m, H40), 1.81 (m, H36b), 1.80 (m, H6b), 1.66 (m, H4b), 1.65 (m, H31b), 1.63 (m, H6a), 1.56 (m, H26b), 1.53 (m, H8b), 1.52 (m, H24a), 1.52 (m, H36a), 1.50 (m, H4a), 1.47 (m, H26a), 1.46 (m, H10b), 1.40 (d, J = 13.9 Hz, H8a), 1.40 (m, H34), 1.26 (m, H32b), 1.22 (m, H10a), 1.13 (m, H32a), 1.08 (m, H31a), 1.03 (m, H22a), 1.01 (s, H59), 0.97 (d, J = 6.9 Hz, H56), 0.83 (d, J = 6.9 Hz, H57), 0.82 (m, H20a), 0.79 (d, J = 7.2 Hz, H54), 0.70 (d, J = 6.6 Hz, H53).

NMR Spectroscopy. NMR measurements were carried out on a Bruker AM-600 NMR spectrometer with an Aspect 3000 computer. COSY and HMBC spectra were recorded in the absolute mode, while ECOSY, NOESY, HOHAHA, ROESY, and HMQC spectra were recorded in the pure phase absorption mode using the time-proportional phase incrementation method. NMR spectra in CD_3OD were measured at 34 °C, while those in DMSO-d_6 were measured at 27 °C. Residual CHD_2OD (3.30 ppm), CD_3OD (49.0 ppm), and CHD_2SOCD_3 (2.49 ppm) signals were used as internal standards. For the spectra measured in CD_3OD, the water resonance was suppressed by presaturation during the relaxation delay. Typically, a total of 450–512 increments of 2K data points was collected for each 2D NMR experiment. For ECOSY spectra, a total of 1024 increments of 4K data points was collected. NOESY spectra were acquired with mixing times of 250 and 700 ms, while ROESY spectra were acquired with a mixing time of 200 ms. The HOHAHA experiments were run with the MLEV-17 pulse sequence for the spin lock interval. The total MLEV-17 spin lock interval was 66 ms, and it was preceded and followed by two purge pulses of 2.5 ms in duration.

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Supplementary Material Available: ^1H NMR spectra of **1** in CD_3OD and DMSO-d_6, COSY, ECOSY, HMQC, ROESY, and HMBC spectra in CD_3OD, and COSY and NOESY spectra in DMSO-d_6 (9 pages). Ordering information is given on any current masthead page.