Plakolide A, a New γ-Lactone from the Marine Sponge *Plakortis* sp.

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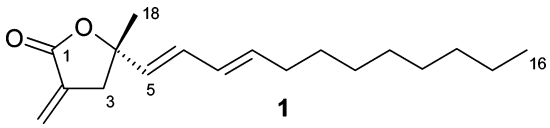
Plakolide A (**1**), a new α-exomethylene-γ-lactone isolated from the marine sponge *Plakortis* sp., was found to inhibit inducible nitric oxide synthase (iNOS) activity. The isolation, structure elucidation, and biological activity of plakolide A is described.

In our continuing search for new enzyme inhibitors from marine organisms,[1] we have isolated a new γ-lactone, trivially named plakolide A, from a shallow-water marine sponge of the genus *Plakortis*. Plakolide A (**1**) showed significant inhibitory activity in a cell-based assay designed to detect inhibitors of inducible nitric oxide synthase (iNOS). Plakolide A possesses an α-exomethylene, γ-disubstituted γ-lactone moiety not previously reported from marine sponges. Recently, a series of related hydroxy compounds possessing an α-exomethylene, β-hydroxy, γ-disubstituted γ-lactone moiety have been reported from a *Plakortis quasiamphiaster* collected in Vanuatu.[2] The genus *Plakortis* is a rich source of stable polyketide-derived peroxides.[3] Some early examples are the branch chain esters plakortin[4] and epiplakortin[5] and related free acids from *P. angulospiculatus*,[6] and the straight-chain metabolites, such as chondrillin[7] and related compounds from *Plakortis lita*[8] and *Plakortis* sp. from the Amirante Islands.[9] A Jamaican specimen of *Plakortis halichondrioides* is a source for both cyclic peroxides, plakortides F−H,[10] and the related bicyclic γ-lactones, plakortones A−D.[11] We report here the details of the isolation, biological activity, and structure determination of a new γ-lactone, plakolide A (**1**), from a *Plakortis* sp. collected from La Palma, Canary Islands.



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The sample of the sponge was collected by hand using scuba in June 1991 and stored at −20 °C until extraction. The EtOH extract of the sponge was partitioned between EtOAc and H_2O. The EtOAc-soluble fraction was chromatographed over a column of Si gel with a gradient of CH_2Cl_2−MeOH, and the fractions were monitored for inhibition of inducible nitric oxide synthase. A fraction that showed inhibition of inducible nitric oxide synthase was rechromatographed over Si gel with a gradient of *n*-hexane−CH_2Cl_2. The iNOS assay active fraction was subjected to HPLC to give plakolide A (3.4 mg, 0.015% wet wt) as a colorless gum.

HRFABMS of plakolide A (**1**) supported the molecular formula $C_{18}H_{28}O_2$ ($[M + H]^+$ *m/z* 277.2116). The UV spectrum displayed the characteristic absorption at λ_{max} 230 nm (log ϵ 4.47) for a conjugated carbonyl group.[8] The IR spectral absorption at 1768 cm^{-1} indicated the presence of a conjugated γ-lactone functionality. The ^1H NMR spectrum in $CDCl_3$ revealed the presence of six olefinic protons at δ 6.21 (1H, dd, *J* = 15.4, 10.4 Hz), 6.20 (1H, t, *J* = 2.4 Hz), 5.97 (1H, dd, *J* = 15.0, 10.4 Hz), 5.73 (1H, dt, *J* = 15.0, 6.9 Hz), 5.58 (1H, d, *J* = 15.4 Hz), and 5.57 (1H, t, *J* = 2.4 Hz); a downfield-shifted methylene group appearing as an AB quartet with allylic coupling at δ 2.89 (1H, dt, *J* = 16.5, 2.4 Hz) and 2.77 (1H, dt, *J* = 16.5, 2.4 Hz); a typical allylic methylene group at δ 2.05 (2H, dt, *J* = 7.2, 6.9 Hz); a methyl singlet attached to an oxygenated carbon at δ 1.50 (3H, s); two sets of methylene groups at δ 1.33 (2H, m) and 1.15 (10H, m); and a terminal methyl triplet at δ 0.85 (3H, t, *J* = 7.2 Hz). The ^{13}C NMR spectrum analyzed together with the DEPT and HMQC spectra revealed signals for three quaternary carbons (δ 169.8, C-1; 135.4, C-2; 82.4, C-4), four olefinic methines, an olefinic methylene, eight methylenes, and two methyl groups. Analysis of the COSY spectrum together with the HMQC data indicated that the exomethylene group at δ 6.20 and 5.57 (^{13}C δ 122.2) is coupled with the methylene group at δ 2.89 and 2.77 (^{13}C δ 40.8), and the remaining four olefinic protons constituted a conjugated diene system (H-5 to H-8). The COSY spectrum also indicated the coupling of the olefinic signal observed at δ 5.73 (H-8) with the allylic methylene at δ 2.05 (H-9), which in turn was coupled to the remaining alkyl chain. The HMBC spectrum of plakolide A in $CDCl_3$ showed three-bond-coupled $^1H-^{13}C$ correlations, H-3 (C-1, C-5, C-17, C-18), H-6 (C-4, C-8), H-7 (C-5, C-9), H-8 (C-6, one of the methylene carbons C-10−C13), H-16 (C-14), H-17 (C-1), and H-18 (C-3, C-5), and thus unambiguously established the substitution at the γ-lactone ring system and confirmed the structure of plakolide A (**1**). The absolute stereochemistry of the C-4Me was assigned using CD data. The CD spectrum (Figure 1) gave a bisignated negatively split curve having a negative first

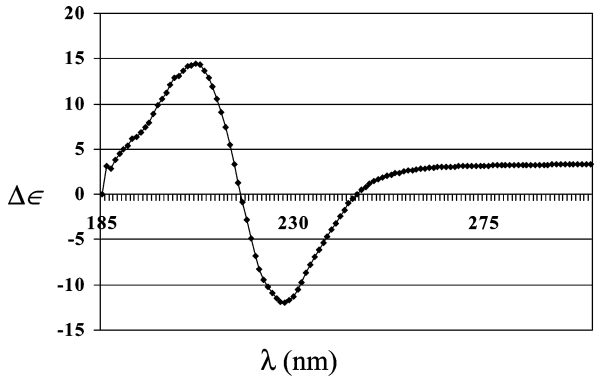

Figure 1. CD spectrum of **1**.

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Cotton effect (228 nm) and a positive second Cotton effect (207 nm). Analysis of these data in the context of chiral exiton coupling[12] of the two chromophores around the C-4 chiral center established 4*S* stereochemistry.

In a cell-based assay designed to detect compounds that inhibit inducible nitric oxide synthase, plakolide A (**1**) gave an apparent inhibition of the production of nitric oxide with an IC_{50} value of 0.2 μg/mL, whereas the concentration decreasing the cell viability by 50% was 1.3 μg/mL. Plakolide A exhibited a 72 h cytotoxicity against the cultured P-388 murine lymphoma and A-549 human lung adenocarcinoma cell lines with IC_{50} values of 1.1 and 5.0 μg/mL, respectively. It also showed cytotoxicity against PANC-1 human pancreatic carcinoma and NCI/ADR human breast carcinoma cell lines with IC_{50} values of 3.8 and 3.7 μg/mL, respectively.[13]

Experimental Section

General Experimental Procedures. Optical rotations were recorded on a Jasco DIP-370 digital polarimeter. The IR spectrum was obtained on a Midac M-1200 instrument with Galactic GRAMS/386 software. The UV spectrum was taken with a Perkin-Elmer Lambda 3B UV/visible spectrophotometer. The CD spectrum was recorded in MeOH using a JASCO J-715 spectropolarimeter. 1D and 2D NMR spectra were measured on a Bruker AMX-500 instrument. The ^{1}H NMR chemical shifts were assigned using a combination of data from COSY and HMQC experiments. Similarly, ^{13}C NMR chemical shifts were assigned on the basis of DEPT and HMQC experiments. The HRMS were obtained on a Finnigan MAT 95Q mass spectrometer at the Spectroscopic Services Group, University of Florida, Gainesville, FL.

Collection and Taxonomy. The sponge sample (HBOI/DBMR # 12-VI-91-3-003) was collected by hand using scuba by one of the authors (J.K.R.) on June 12, 1991, from the wall of a cave at a depth of 3.3 m, near La Palma, Canary Islands (latitude 28°41.75′ N; longitude 17°58.00′ W). This specimen most closely fits the genus *Plakortis* Schulz, 1880 (class Demospongia, order Homosclerophorida, family Plakinidae), but cannot be ascribed to a known species at this time. The specimen was encrusting on the rock surface of a cave wall. It was ~15 cm in diameter and ~1 cm thick, with a smooth ectosome. The color in situ was light brown. The spicule skeleton consists of diods and occasional triods. A museum voucher specimen preserved in EtOH has been deposited at the Harbor Branch Oceanographic Museum, catalog number 003:00976 and is available for taxonomic evaluation.

Extraction and Isolation. The sponge (12-VI-91-3-003, 200 g wet wt) was extracted in EtOH (3 × 300 mL) and concentrated to give 9.3 g of the EtOH extract. The EtOH extract was partitioned between EtOAc and H_2O. The EtOAc-soluble fraction (1.8 g) was chromatographed on a column of Si gel (230−400 mesh) using a CH_2Cl_2−MeOH step gradient, and the fractions were monitored for inhibition in the iNOS assay. The active fraction that eluted with CH_2Cl_2 was rechromatographed over Si gel using a *n*-hexane−CH_2Cl_2 step gradient, and the fractions were re-monitored for inhibition in the iNOS assay. The active fraction (0.037 g) that eluted with 25% *n*-hexane−CH_2Cl_2 on further purification by HPLC (Phenomenex, SiO_2, 5 μm, 250 × 10 mm) with a *n*-hexane−EtOAc gradient (95:5 to 80:20 in 16 min, 3 mL/min) gave plakolide A (**1**) as a colorless gum (3.4 mg, 0.015% of wet wt).

Plakolide A (1): $[\alpha]^{24}_D$ −41° (*c* 0. 1, CH_3OH); UV (MeOH) λ_{max} 208 (log ϵ 4.36), 230 (4.47) nm; IR (NaCl disk) ν_{max} 2934, 2858, 1768, 1457, 1380, 1269, 1208, 1108, 1051, 993, 941 cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$ referenced at δ 7.24) δ 6.21 (1H, dd, J = 15.4, 10.4 Hz, H-6), 6.20 (1H, t, J = 2.4 Hz, H-17), 5.97 (1H, dd, J = 15.0, 10.4 Hz, H-7), 5.73 (1H, dt, J = 15.0, 6.9 Hz, H-8), 5.58 (1H, d, J = 15.4 Hz, H-5), 5.57 (1H, t, J = 2.4 Hz, H-17), 2.89 (1H, dt, J = 16.5, 2.4 Hz, H-3), 2.77 (1H, dt, J = 16.5, 2.4 Hz, H-3), 2.05 (2H, dt, J = 7.2, 6.9 Hz, H-9), 1.50 (3H, s, H-18), 1.33 (2H, m, H-10), 1.15 (10H, m, H11−H15), 0.85 (3H, t, J = 7.2 Hz, H-16); ^{13}C NMR (125.7 MHz, $CDCl_3$ referenced at δ 77.0) δ 169.8 (qC, C-1), 137.4 (CH, C-8), 135.4 (qC, C-2), 132.2 (CH, C-5), 129.7 (CH, C-6), 128.7 (CH, C-7), 122.2 (CH_2, C-17), 82.4 (qC, C-4), 40.8 (CH_2, C-3), 32.7 (CH_2, C-9), 31.9 (CH_2, C-14), 29.4, 29.3, 29.2, 29.1 (CH_2, C-10−C13), 27.1 (CH_3, C-18), 22.7 (CH_2, C-15), 14.1 (CH_3, C-16); HRFABMS (thioglycerol) *m/z* 277.2116 $[M + H]^+$ (calcd for $C_{18}H_{29}O_2$, 277.2067).

Determination of iNOS Activity. Fractions and compounds were diluted in ethanol, aliquoted into appropriate wells of a 96-well microtiter plate, and allowed to air-dry for 1 h at room temperature. RAW 264.7 cells (ATCC TIB-71 mouse monocyte-macrophage cell line derived from Abelson leukemia virus transformed cells) were then subcultured into each well at a density of 5×10^4 cells/well in 200 μL of tissue culture medium (TCM) containing lipopolysaccharide (1 ng/mL; Sigma) and interferon-γ (2 units/mL; PharMingen, San Diego, CA). Plates were incubated at 37 °C, 5% CO_2 for 24 h. A 150 μL sample of the TCM from each well was transferred to a second microtiter plate, 100 μL Griess reagent[14] was added to this TCM, and the color intensity of the reaction was determined by spectrophotometric absorbance at 540 nm (Spectra II; Tecan US Inc, Durham, NC).

Viability of the cells was determined by the methods of Mossman.[15] Briefly, 150 μL of TCM containing 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT, 2.5 mg/mL; Sigma) was added to each well containing the remaining 50 μL volumes of RAW 264.7 cells and incubated at 37 °C, 5% CO_2, for 3 h. The medium was removed from all wells and replaced with 200 μL of acidified isopropyl alcohol (0.2% HCl). The plates were shaken for 15 min, and the presence of the blue formazan was detected using a Spectra II plate reader set at 570 nm and with a 650 nm reference filter. Viability of other cell lines was determined following their 72 h incubation with plakolide A and using the same MTT method as described above.

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References and Notes

(1) Gunasekera, S. P.; McCarthy, P. J.; Longley, R. E.; Pomponi, S. A.; Wright, A. E. *J. Nat. Prod.* **1999**, *62*, 1208−1211, and references therein.
(2) Zampella, C.; Giannini, C.; Debitus C.; D'Auria, M. V. *Tetrahedron* **2001**, *57*, 257−263.
(3) Faulkner, D.J. *Nat. Prod. Rep.* **2002**, *19*, 1−48, and references therein.
(4) Higgs, M. D.; Faulkner, D. J. *J. Org. Chem.* **1978**, *43*, 3454−3457.
(5) Stierle, D. B.; Faulkner, D. J. *J. Org. Chem.* **1979**, *44*, 964−968.
(6) Gunasekera, S. P.; Gunasekera, M.; Gunawardana, G. P.; McCarthy, P.; Burres, N. *J. Nat. Prod.* **1990**, *53*, 669−674.
(7) Sakemi, S.; Higa, T.; Anthoni, U.; Christophersen, C. *Tetrahedron* **1987**, *43*, 263−268.
(8) Harrison, B.; Crews, P. *J. Nat. Prod.* **1998**, *61*, 1033−1037.
(9) Braekman, J. C.; Daloze, D.; Groote, S. De; Fernandes, J. B.; van Soest, R. W. M. *J. Nat. Prod.* **1998**, *61*, 1038−1042.
(10) Patil, A. D.; Freyer, A. J.; Carte, B.; Johnson, R. K.; Lahouratate, P. *J. Nat. Prod.* **1996**, *59*, 219−223.
(11) Patil, A. D.; Freyer, A. J.; Bean, M. F.; Carte, B.; Westley, J. W.; Johnson, R. K.; Lahouratate, P. *Tetrahedron* **1996**, *52*, 377−394.
(12) Hirada, N.; Nakanishi, K. *Circular Dichroic Spectroscopy: Exciton Coupling in Organic Stereochemistry*; University Science Books: Mill Valley, CA, 1983; Chapter 1, pp 1−31.
(13) Gunasekera, S. P.; Longley, R E.; Pomponi, S. A.; Wright, A. E.; Reed, J. K. Serial number 10/382,369, U.S. Patent filed on March 6, 2003.
(14) Greenberg, S. S.; Xie, J.; Spitzer, J. J.; Wang, J. F.; Lancaster, J.; Grisham, M. B.; Powers, D. R.; Giles, T. D. *Life Sci.* **1995**, 57, 1949−1961.
(15) Mossman, T. *J. Immunol. Methods* **1983**, 65, 55−63.

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