Discorhabdin P, a New Enzyme Inhibitor from a Deep-Water Caribbean Sponge of the Genus *Batzella*

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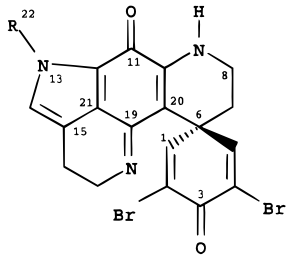
Discorhabdin P (**1**), a new discorhabdin analogue, has been isolated from a deep-water marine sponge of the genus *Batzella*. Discorhabdin P (**1**) inhibited the phosphatase activity of calcineurin and the peptidase activity of CPP32. It also showed in vitro cytotoxicity against P-388 and A-549 cell lines. The isolation and structure elucidation of discorhabdin P (**1**) are described.

Marine sponges continue to be a rich source of secondary metabolites with novel structures and desirable biological activities.[1] In a continuing search for new protein phosphatase inhibitors from marine organisms,[2] we have isolated a new discorhabdin analogue that inhibits calcineurin (CaN), from a deep-water marine sponge of the genus *Batzella*. The compound also inhibits the peptidase activity of CPP32. The inhibitor, trivially named discorhabdin P (**1**), is *N*-methyldiscorhabdin C and has not been previously described in the literature. The structure was determined by a combination of NMR spectral studies and single-crystal X-ray diffraction. Discorhabdin C (**2**), the first compound in this series was reported from the sponge of the genus *Latrunculia* du Bocage by a New Zealand group in 1986.[3] Since then, the same group[4−8] and others[9−11] have published on discorhabdins A−O, and the discorhabdin compounds all possess an iminoquinone and a spiro-enone or spiro-dienone system. These compounds, many of which are biologically active, are believed to be biosynthesized from a substituted tyrosine and a tryptamine derivative.



(**1**) R = CH_3

(**2**) R = H

Calcineurin is a serine/threonine protein phosphatase involved in signal transduction and is recognized as being one of the principal signaling molecules that regulates immune responsiveness.[12] Immunosuppressants such as FK506 and cyclosporin A have been shown to exert their effect through inhibition of CaN following their association with binding proteins.[13] This finding has prompted a search for small-molecule inhibitors of CaN, which might be expected to have useful pharmacologic activity.

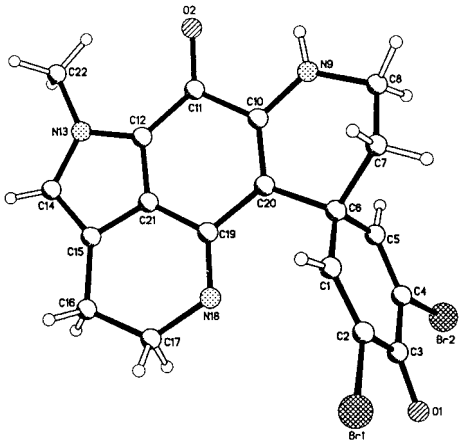


Figure 1. A computer generated perspective drawing of the final X-ray model of discorhabdin P (**1**).

The caspases, which include CPP32, are a group of at least 10 cysteine proteases (also known as interleukin-2 converting enzymes or ICE_2), which play a major role in the programmed cell-death mechanism known as apoptosis.[14] These enzymes are the mammalian homologues of the ced-3 gene product that modulates apoptotic processes in the nematode *Caenorhabditis elegans*.[15] Mutations in ced-3 prevent apoptosis during normal development of the nematode and in mammals, and inhibitors of caspase-3 (CPP32) have been shown to prevent apoptotic-mediated death in a number of cell lines and in various tissues.[15,16]

Apoptotic mechanisms play important roles in the normal development of the immune repertoire and in tissue remodeling during embryonic development in both vertebrates and invertebrates.[17] However, aberrant apoptosis has been implicated in a number of experimental and human disease states. For example, in acute CNS injury following hypoxic−ischemic insult in mice, there is evidence that caspase-induced apoptosis is the prime factor in neuronal destruction.[18] In addition, caspases have been implicated in chronic neurodegenerative disorders, which would suggest their role in the pathogenesis of amyotrophic lateral sclerosis (ALS) and in Alzheimer's disease.[19,20] Caspase-3 (CPP32) has also been shown to be involved in the stimulation of IL-8 secretion of synoviocytes in rheumatoid arthritis, which serves to increase joint inflammation and progress of the disease.[21] Therefore, inhibitors of caspase enzymatic activities may serve to prevent the

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pathological damage induced by caspase-mediated apoptotic events.

The sponge specimen was collected on August 24, 1994, by manned submersible from the western Great Bahama Bank, Bahamas, and was stored at −20 °C until extraction. The specimen was soaked in EtOH and the concentrated EtOH extract partitioned between EtOAc and H_2O. The EtOAc-soluble fraction was chromatographed over Si gel with CH_2Cl_2−MeOH and fractions monitored for inhibition of CaN. The fraction that showed the greatest inhibition of CaN was rechromatographed over Si gel with CH_2Cl_2−MeOH to give discorhabdin P (**1**) as an orange solid. Crystallization of **1** from 5% MeOH−CH_2Cl_2 gave orange crystals (yield, 0.015% of wet wt).

HRFABMS supported the molecular formula $C_{19}H_{16}Br_2N_3O_2$ [(M + H^+) *m/z* 475.978, 477.968, 479.968, Δ 5 mmu]. The UV spectrum displayed characteristic absorptions at λ_{max} 245 (log ϵ 4.28) and 337 (4.00) nm of a cross-conjugated pyrrolo[1,7]phenanthroline chromophore, as reported for makaluvamine A.[22] IR spectral absorptions at 1667 and 1653 cm^{-1} indicated the presence of a conjugated carbonyl functionality. The ^1H NMR spectrum revealed the presence of a 2H olefinic singlet (δ 8.17), a 1H olefinic singlet (δ 7.45), an *N*-methyl singlet (δ 4.27), and signals for four methylene groups (δ 4.18, 3.84, 2.91, 2.30). Analysis of the COSY spectrum indicated that the four methylene groups constituted two isolated spin systems identical to those reported for discorhabdin C.[3] The ^{13}C NMR spectrum analyzed together with the DEPT spectrum revealed signals for 11 quaternary carbons, three olefinic doublets (two of which overlapped), four methylene triplets, and a methyl quartet. These data suggested that the compound is a member of the discorhabdin series. Because of the absence of NH signals in the ^1H NMR spectrum and the lack of any HMBC correlations to the C-11 carbonyl group,[23] the compound was recrystallized for X-ray studies, and the structure of discorhabdin P (**1**) was confirmed by single-crystal X-ray diffraction.

The structure was solved by direct methods and refined using the SHELXTL (version 5.0) software package. The final anisotropic full-matrix least-squares refinement on F^2 (H-atoms in calculated positions) converged at R_1 = 3.91%, wR_2 = 10.85% with a goodness-of-fit of 1.049. The largest peak on the final difference map was 0.62e/Å^3.

In the free base structure of discorhabdin P, the difference in the N(9)−C(10) and N(18)−C(19) bond distances indicate that the *p*-imino−quinone tautomer is preferred in the solid state. The flat C(15)−C(16)−C(17)−N(18) system coupled with the anomalously short C−(16)−C(17) distance of 1.48 Å suggests that the two methylenes are disordered—a suggestion supported by their relatively high thermal parameters. There is one CH_2Cl_2 solvent molecule per asymmetric unit, and archival X-ray data have been deposited in the Cambridge Crystallographic Data Center.[24]

Experimental Section

General Experiment Procedures. The IR spectrum was obtained on a Midac FTIR M 1200 instrument. The UV spectrum was obtained with a Perkin−Elmer Lambda 3B UV/vis spectrophotometer. 1D and 2D NMR spectra were measured on a Bruker AMX-500 instrument. The ^1H 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 was obtained on a Finnigan MAT95Q mass spectrometer at the Spectroscopic Services Group, University of Florida.

Collection and Taxonomy. The sponge sample (HBOI no. 24-VIII-94-3-001) was collected in August 1994, by manned submersible (Clelia dive number 303) at a depth of 141 m, from the western Great Bahama Bank, Bahamas (latitude 25°15.562′ N; longitude 79° 11.109′ W). This sponge has been assigned to the genus *Batzella* (class Demospongia, order Poecilosclerida, family Desmacididae), as described and discussed by Van Soest et al.[25] The sponge has a detachable ectosome and a spicule skeleton of strongyles of one size category. Some of the strongyles have malformed tips. The sponge incorporates sediment into its skeleton. There are numerous fistules scattered over the surface of the sponge. The sponge is purple-brown when alive, brown when preserved in EtOH. A taxonomic reference sample has been deposited in the Harbor Branch Oceanographic Museum, catalogue no. 003:00922.

Extraction and Isolation. The sponge (wet wt 102 g) was soaked in EtOH and the concentrated EtOH extract (4 g) was partitioned between EtOAc and H_2O. The EtOAc-soluble fraction (0.6 g) was then column chromatographed over Si gel (230−400 mesh) using a CH_2Cl_2−MeOH step gradient and was monitored for inhibition of the CaN protein phosphatase. The fractions that showed the greatest inhibition of CaN were combined and rechromatographed over Si gel with a CH_2Cl_2−MeOH step gradient to give discorhabdin P (**1**) as an orange solid. Crystallization of **1** from 5% MeOH−CH_2Cl_2 afforded orange crystals (yield, 0.015% of wet wt).

Discorhabdin P (1): mp >360 °C, blackened at 162 °C; UV (MeOH) λ_{max} 488 (log ϵ 3.49), 337 (4.00), 245 (4.28), 200 (4.26) nm; IR (KBr) ν_{max} 3385, 3049, 2930, 2858, 2363, 1666, 1653, 1569, 1530, 1438, 1330, 1291, 1023, 696 cm^{-1}; ^1H NMR (DMSO-d_6, 500 MHz) δ 8.17 (2H, s, H-1, H-5), 7.45 (1H, s, H-14), 4.27 (3H, s, H-22), 4.18 (2H, t, J = 7.8 Hz, H-17), 3.84 (2H, t, J = 5.5 Hz, H-8), 2.91 (2H, t, J = 7.8 Hz, H-16), 2.30 (2H, t, J = 5.5 Hz, H-7); ^{13}C NMR (DMSO-d_6, 125.7 MHz) δ 172.4 (s, C-3), 170.0 (s, C-11), 157.3 (d, C-1), 157.3 (d, C-5), 153.7 (s, C-19), 143.2 (s, C-10), 129.0 (d, C-14), 121.5 (s, C-12), 121.5 (s, C-21), 118.4 (s, C-2), 118.4 (s, C-4), 116.3 (s, C-15), 99.7 (s, C-20), 49.5 (t, C-17), 46.4 (s, C-6), 36.6 (t, C-8), 35.0 (q, C-22), 32.3 (t, C-7), 17.5 (t, C-16); HRFABMS (3-nitrobenzyl alcohol) *m/z* 475.968, 477.968, 479.968, Δ 5 mmu for $C_{19}H_{16}Br_2N_3O_2$ [M^+ + H].

Single-Crystal X-ray Structure Determination. A small crystal of discorhabdin P (**1**), $C_{19}H_{15}Br_2N_3O_2$, with approximate dimensions 0.3 × 0.2 × 0.15 mm^3 was selected for X-ray crystallographic analysis. The X-ray intensity data were measured at room temperature on a Bruker SMART CCD-based X-ray diffractometer using Mo Kα (λ = 0.71073 Å) radiation. The detector to crystal distance was 4.5 cm.

A total of 2560 frames was collected with a scan width of 0.3° in ω and an exposure time of 30 s/frame. The frames were integrated with the SAINT software package using a narrow-frame integration algorithm. Integration yielded a total of 4727 reflections to a maximum 2θ angle of 46°, of which 2939 were symmetry independent (R_{int} = 1.91%) and 2536 were greater than 4σ(F). The final triclinic cell constants of a = 8.203(1), b = 9.399(1), c = 14.647(1) Å, α = 102.44 (1), β = 104.18(1), γ =96.38(1), v = 1053.0(2) Å^3, are based upon the refinement of the *xyz*-centroids of 3421 reflections above 20σ(I). Analysis of the data showed negligible decay during data collection.

Calcineurin Assay. Calmodulin was prepared from bovine brain according to the method of Wallace et al.[26] The calmodulin was either used directly or coupled to sepharose-4B to form the calmodulin−sepharose affinity column necessary for the isolation of calcineurin. Calcineurin was prepared from bovine brain by the method of Tallant et al.,[27] concentrated, aliquoted, and stored at −80 °C.

Calcineurin activity was assayed in 96-well microtiter plates in a final volume of 50 μL. Each well contained 50 mM Tris−HCl, pH 7.5, 0.5 mM $MnCl_2$, 0.05 mM $CaCl_2$, 1 mM DTT, 50 mM *p*-nitrophenyl phosphate (pNPP), 0.3 μg calcineurin, a five-fold excess of calmodulin, and either test sample or control compound. Plates were incubated at 30 °C for 60 min. Liberated *p*-nitrophenol was determined by the change in absorbance at 405 nm.

CPP32 Assay. Test samples were aliquoted into 96-well microtiter plates and allowed to air-dry. The stock CPP32 enzyme (kindly supplied by BASF, Worcester, MA) was diluted by adding 10 μL of the enzyme to 17 mL of reaction buffer (50 mM HEPES pH 7.5; 10% glycerol; 5 mM DTT; 0.5 mM EDTA). A volume of 180 μL of the diluted enzyme was added to wells containing the dried test samples, or to empty wells (control). The contents of the microtiter wells were mixed by shaking on a plate shaker. Plates were incubated at 30 °C for 5 min. A volume of 20 μL of substrate (Asp-Glu-Val-Asp-CHO) was added to each well, which resulted in a final concentration of 25 μM. Controls for each plate consisted of an inhibitor control (50 nM (*N*-acetyl-Asp-Glu-Val-Asp-*p*-nitroanilide) final concentration); positive control (enzyme and substrate) and negative control (reaction buffer and substrate). The plates were covered with aluminum foil and mixed using a plate shaker for 5 min then incubated at 30 °C for 30 min. Plates were read on a plate reader with absorbance measured at 405 nM. Data were expressed as percent inhibition by comparing the absorbance values of test samples with those of the positive control (no sample). The IC_{50} determination was defined as the concentration of sample/pure compound that resulted in a 50% inhibition of the enzyme−substrate absorbance value.

Biological Activity. Discorhabdin P (**1**) inhibited CaN and CPP32 with IC_{50} values of 0.55 and 0.37 μg/mL, respectively.[28] Positive controls for calcineurin and CPP32 assays consisted of the addition of both substrate and enzyme in the absence of test compounds. More importantly, the inhibitor controls include 500 μM of sodium *ortho*-vanadate for the calcineurin assay (consistently, 70% inhibition with this inhibitor), and the substrate analogue, Asp-Glu-Val-Asp-CHO for the CPP32 assay (50.0 nM, which gives 90−100% inhibition). Both levels of inhibition are highly significant, as there are presently only a handful of compounds in the literature that demonstrate nM potency for inhibition of either calcineurin or CPP32 enzyme activity. The parent compound discorhabdin C (**2**) from our in-house depository indicated no activity against CPP32 and calcineurin enzymes at the tested highest dose of 5.0 μg/mL.

Compound **1** also exhibited in vitro cytotoxicity against the cultured murine P-388 tumor cell line and human lung carcinoma A-549 cell line, with IC_{50} values of 0.025 and 0.41 μg/mL, respectively.

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