Discorhabdins S, T, and U, New Cytotoxic Pyrroloiminoquinones from a Deep-Water Caribbean Sponge of the Genus *Batzella*

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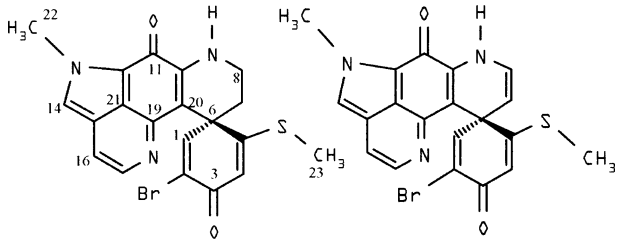
Discorhabdins S, T, and U (**1**−**3**), three new discorhabdin analogues, have been isolated from a deep-water marine sponge of the genus *Batzella*. These discorhabdin analogues showed in vitro cytotoxicity against PANC-1, P-388, and A-549 cell lines. The isolation and structure elucidation of discorhabdins S, T, and U are described.

Marine sponges belonging to the genus *Batzella* are a promising source of pyrroloiminoquinone alkaloids.[1] Recently, we reported the isolation, structure elucidation, and biological activities of discorhabdin P from the sponge genus *Batzella*.[2] Subsequently, the National Cancer Institute group[3] in Maryland and the University of Melbourne group[4] in Australia have reported the isolation of discorhabdins Q and R, respectively. Since the first report of discorhabdin C by Perry et al. in 1986,[5] more than 60 compounds containing the pyrroloiminoquinone moiety have been isolated from the sponges of the genera *Latrunculia*,[3−8] *Prianos*,[6] *Zyzzya*,[3,9−12] *Histodermella*,[13] and *Batzella*.[2,14] In a continuing search for new cytotoxic compounds from marine organisms, we have isolated three new discorhabdin analogues, which are cytotoxic to PANC-1, P-388, and A-549 cell lines, from a deep-water marine sponge of the genus *Batzella*. The new compounds, trivially named discorhabdins S, T, and U (**1**−**3**), are C-5 *S*-methyl discorhabdin P analogues and have not been previously described in the literature. The structures were determined by a combination of NMR and mass spectral studies and by comparison with the NMR data of related compounds reported in the literature.



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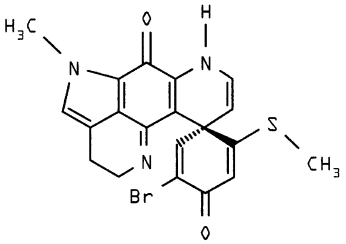

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The sponge sample was collected in October 2000 by manned submersible from Ocean Cay, south of Bimini, Bahamas, and was stored at −20 °C until extraction. The specimen was soaked in EtOH, and the EtOH extract after concentration was partitioned between EtOAc and H_2O. The EtOAc-soluble fraction was chromatographed over silica gel using CH_2Cl_2−MeOH as eluent, and fractions were monitored for discorhabdin analogues by ^{1}H NMR and TLC analyses. The colored chromatography fractions that showed characteristic ^{1}H NMR spectral patterns described for discorhabdin P[2] were further separated to give dark orange compounds identified as discorhabdin S (10.0 mg yield, 0.005% of wet wt), discorhabdin T (2.0 mg yield, 0.001% of wet wt), and discorhabdin U (5.0 mg yield, 0.002% of wet wt).

High-resolution LSIMS of discorhabdin S (**1**) supported the molecular formula $C_{20}H_{16}BrN_3O_2S$ ($[M + H]^+$ *m/z* at 442.0261, 444.0470). The UV spectrum displayed characteristic absorptions at λ_{max} 422 (log ϵ 4.14), 400 (3.94), 305 (4.04), 260 (4.27), and 225 (4.54) nm for a cross-conjugated iminoquinone.[14] A strong IR absorption at 1637 cm^{-1} indicated the presence of a cross-conjugated carbonyl functionality. The ^{1}H NMR spectrum revealed the presence of two olefinic doublets (δ 8.08, 7.15, J = 5.8 Hz), three olefinic singlets (δ 7.54, 7.50, 6.14), an *N*-methyl singlet (δ 4.19), an *S*-methyl singlet (δ 2.15), and signals for two mutually coupled methylene groups [δ 3.73, 3.46 (2H, m); 2.23, 2.02 (2H, m)]. Analysis of the COSY spectrum indicated that the two olefinic doublets are coupled to each other and the two methylene groups constituted an isolated spin system similar to that reported for discorhabdin P.[2] The diastereotopic chemical shift values of the two methylene groups indicated the effect of the neighboring *S*-methyl group.[3] The comparison of 7-H_2 chemical shift values reported for discorhabdins B[7] and Q[3] enabled the assignment of the diastereotopic chemical shift values 2.23 and 2.02 ppm in **1** to 7-βH and 7-αH, respectively. The ^{13}C NMR spectrum analyzed together with the DEPT and HMQC spectra revealed signals for 11 quaternary carbons, five olefinic methines, two methylenes, and two methyl carbons. The three low-field quaternary carbon signals appearing at 176.6, 175.0, and 165.9 ppm were tentatively assigned to C-5, C-3, and C-11, respectively, by comparison with the data reported in the literature.[2,3] These ^{13}C NMR data together with the presence of the characteristic discorhabdin-spirocarbon at 47.5 ppm established that the compound is a member of the discorhabdin series. The HMBC spectrum of discorhabdin S showed three-bond-

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coupled $^1H-^{13}C$ correlations H-22 (C-12, C-14), H-14 (C-12, C-16, C-21), H-16 (C-14, C-21), and H-17 (C-15, C19), which established the position of the *N*-methyl group and the two vicinally coupled olefinic doublets. Similarly, the HMBC spectrum showed three-bond-coupled $^1H-^{13}C$ correlations, H_2-8 (C-10, C-6) and H_2-7 (C-1, C-5, C-20), and these data together with the chemical shift values of H_2-8 (δ 3.73, 3.46) and H_2-7 (δ 2.23, 2.02) confirmed the position of the two methylene groups in the molecule. Additional three-bond-coupled $^1H-^{13}C$ correlations, H-1 (C-3, C-5, C-7), H-4 (C-2, C-6), H_2-7 (C-1, C-5, C-20), and H-23 (C-5), supported the position of the carbonyl (δ 175.0) at C-3, connected the *S*-methyl group to C-5, and placed H-1 and H-4 on the opposite side of the cross-conjugated ketone. These data allowed us to assign the bromine to the remaining quaternary C-2. The NOE observed between H-4 and *S*-Me confirmed the above assignment. Further, a strong NOE observed between H-1 and H-7α indicated the relative configuration at the spiro-carbon C-6, and therefore it has the same configuration reported for discorhabdins B and Q.[4] The combination of the above data established the structure for discorhabdin S (**1**).

High-resolution EIMS analysis of discorhabdin T (**2**) was consistent with the molecular formula $C_{20}H_{14}BrN_3O_2S$ (M^+ *m/z* at 438.9996, 440.9869) and indicated a difference in elements H_2 (2 mmu) from discorhabdin S (**1**). The UV and IR spectra of discorhabdin T closely resembled those of discorhabdin S (**1**). The ^1H NMR spectrum showed similarity to that of discorhabdin S and revealed the presence of three olefinic singlets (δ 7.64, 7.62, 5.91), an *N*-methyl singlet (δ 4.25), an *S*-methyl singlet (δ 2.18), two olefinic doublets (δ 8.17, 7.30, J = 5.8 Hz), and a second pair of olefinic doublets (δ 6.43, 4.10, J = 7.5 Hz) in place of the two mutually coupled methylene groups in discorhabdin S. The COSY spectrum established the coupling of the signals at 8.17 to 7.30 ppm and 6.43 to 4.10 ppm. The ^{13}C NMR spectrum analyzed together with the DEPT and HMQC spectra revealed signals for 11 quaternary carbons, seven olefinic methines, and two methyl carbons. The three low-field quaternary carbon signals appearing at 177.0, 176.5.0, and 165.1 ppm were assigned to C-5, C-3, and C-11, respectively, by comparison with the data reported for discorhabdin S. The HMBC spectrum of discorhabdin T showed three-bond-coupled $^1H-^{13}C$ correlations H-8 (δ 6.43) to (C-6, C-10) and H-7 (δ 4.10) to (C-1, C-5, C-20) and all correlations reported for discorhabdin S. These data established the structure of discorhabdin T as the 7,8-dehydrodiscorhabdin S (**2**).

High-resolution LSIMS of discorhabdin U (**3**) established the molecular formula $C_{20}H_{16}BrN_3O_2S$ ($[M + H]^+$ *m/z* at 442.0275, 444.0052) and revealed that it is an isomer of discorhabdin S. The UV, IR, and NMR spectra of discorhabdin U closely resembled the spectra of discorhabdin S (**1**). The ^1H NMR spectrum revealed the presence of two mutually coupled olefinic doublets (δ 6.38, 4.09, J = 7.5 Hz), three olefinic singlets (δ 7.57, 6.63, 5.88), an *N*-methyl singlet (δ 3.90), an *S*-methyl singlet (δ 2.29), and signals for two mutually coupled methylene groups (δ 3.93, t, J = 8.0 Hz; 2.58, t, J = 8.0 Hz). The signals for the two methylene groups (δ 3.93, 2.58) did not show any diastereotropism, as seen in discorhabdin S, and thus suggested that these groups are positioned away from the spiro-carbon and its adjacent *S*-methyl ether group. Furthermore, these two methylene groups were shifted downfield, signifying that they contribute to the pyrroloiminoquinone ring system as reported for discorhabdin P. The ^{13}C NMR spectrum analyzed together with the DEPT and HMQC spectra showed signals for 11 quaternary carbons, five olefinic methines, two methylenes, and two methyl carbons. The three low-field quaternary carbon signals at 176.6, 175.2, and 169.4 ppm were assigned to C-5, C-3, and C-11, respectively, and the high-field quaternary carbon at 49.7 ppm by comparison with **1** and **2** was assigned to the spiro-carbon C-6. The HMBC spectrum showed three-bond-coupled $^1H-^{13}C$ correlations H-22 (C-12, C-14), H-14 (C-12, C-16, C-21, C22), H_2-16 (C-14, C-21), and H_2-17 (C-15, C19), which established the position of the *N*-methyl group and the two vicinally coupled methylene triplets. Similarly, the HMBC spectrum showed three-bond-coupled $^1H-^{13}C$ correlations H-8 (C-10, C-6) and H-7 (C-1, C-5, C-20), and these data together with the chemical shift values of H-8 (δ 6.38) and H-7 (δ 4.09) confirmed the position of the vicinally coupled two olefinic doublets in the molecule. Additional three-bond-coupled $^1H-^{13}C$ correlations, H-1 (C-3, C-5, C-7), H-4 (C-2, C-6), H-7 (C-1, C-5, C-20), and H-23 (C-5), supported the position of the carbonyl (δ 175.0) at C-3, *S*-methyl group at C-5, and placed H-1 and H-4 on the opposite side of the cross-conjugated ketone. These data unambiguously assigned the sulfur atom to C-5 and the bromine atom to the quaternary C-2 as in compounds **1** and **2**. The combination of these data established the structure for discorhabdin U (**3**).

Experimental Section

General Experiment Procedures. UV spectra were measured with a Hitachi U-3010 spectrophotometer. IR spectra were obtained on a Midac M-1200 with Galactic GRAMS/386 software. 1D and 2D NMR spectra were measured on a Bruker AMX-500 instrument. The ^1H NMR chemical shifts (referenced to $CDCl_3$ observed at 7.24 ppm) were assigned using a combination of data from COSY and HMQC experiments. Similarly, ^{13}C NMR chemical shifts (referenced to $CDCl_3$ observed at 77.0 ppm) were assigned on the basis of DEPT and HMQC experiments. The HRMS were obtained on a Finnigan MAT95Q mass spectrometer at the Spectroscopic Services Group, University of Florida, Gainesville, FL.

Collection and Taxonomy. The sponge sample (HBOI # 22-X-00-1-002) was collected on October 22, 2000, by manned submersible (JSL I-4272) at a depth of 141 m, from the Ocean Cay, approximately 20 nautical miles south of Bimini, Bahamas (latitude 25°23.93′ N; longitude 79°14.37′ W). This sponge has been assigned to the genus *Batzella* (class Demospongiae, order Poecilosclerida, family Desmacidonidae), as described and discussed by Van Soest et al.[15] 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 dark brown to black when alive, brown when preserved in ethanol. A taxonomic reference sample has been deposited in the Harbor Branch Oceanographic Museum, catalog number 003:00983.

Extraction and Isolation. The sponge (wet wt 200 g) was soaked in EtOH, and the concentrated EtOH extract (5.27 g) was partitioned between EtOAc and H_2O. The EtOAc-soluble fraction (0.7 g) was column chromatographed over silica gel (230−400 mesh) using a CH_2Cl_2−MeOH step gradient and monitored for discorhabdin analogues by ^1H NMR and TLC analysis. The colored fractions that showed the characteristic ^1H NMR pattern described for discorhabdin P were further separated by preparative TLC (SiO_2, 20 × 20 cm, thickness 0.5 mm) with 1% MeOH−CH_2Cl_2 to give dark orange compounds, which were designated as discorhabdin S (**1**, 10.0 mg yield, 0.005% of wet wt), discorhabdin T (**2**, 2.0 mg yield, 0.001% of wet wt), and discorhabdin U (**3**, 5.0 mg yield, 0.002% of wet wt).

Discorhabdin S (1): UV (MeOH) λ_{max} 422 (log ϵ 4.14), 400 (3.94), 305 (4.04), 260 (4.27), 225 (4.54) nm; IR (NaCl, neat)

ν_{max} 3385, 3043, 2924, 1637, 1602, 1543, 1498, 1348, 1318, 1240, 1017, and 735 cm^{-1}; ^1H NMR (10% $CD_3OD/CDCl_3$) δ 8.08 (1H, d, J = 5.8 Hz, H-17), 7.54 (1H, s, H-14), 7.50 (H, s, H-1), 7.15 (1H, d, J = 5.8 Hz, H-16), 6.14 (1H, s, H-4), 4.19 (3H, s, H-22), 3.73, 3.46 (2H, m, H-8), 2.23, 2.02 (2H, m, H-7), 2.15 (3H, s, H-23); ^{13}C NMR (10% $CD_3OD/CDCl_3$) δ 176.6 (qC, C-5), 175.0 (qC, C-3), 165.9 (qC, C-11), 155.5 (CH, C-1), 146.1 (qC, C-19), 145.9 (qC, C-10), 142.3 (CH, C-17), 130.6 (CH, C-14), 123.5 (qC, C-15), 122.4 (qC, C-2), 119.1 (qC, C-12), 118.6 (qC, C-21), 117.7 (CH, C-4), 111.0 (CH, C-16), 104.7 (qC, C-20), 47.5 (qC, C-6), 37.9 (CH_2, C-7), 37.2 (CH_3, C-22), 36.9 (CH_2, C-8), 14.7 (CH_3, C-23); HRLSIMS (3-nitrobenzyl alcohol) m/z at 442.0261, 444.0470 $[M + H]^+$ (calcd for $C_{20}H_{17}{}^{79}BrN_3O_2S$, 442.0225, $C_{20}H_{17}{}^{81}BrN_3O_2S$, 444.0434).

Discorhabdin T (2): UV (MeOH) λ_{max} 432 (log ϵ 3.65), 412 (3.55), 305(3.65), 245 (3.97), 225 (4.11) nm; IR (NaCl, neat) ν_{max} 3375, 2874, 1637, 1609, 1554, 1476, 1315, 1265, 1093, and 805 cm^{-1}; ^1H NMR (10% $CD_3OD/CDCl_3$) δ 8.17 (1H, d, J = 5.8 Hz, H-17), 7.64 (1H, s, H-14), 7.62 (H, s, H-1), 7.30 (1H, d, J = 5.8 Hz, H-16), 6.43 (1H, d, J = 7.5 Hz, H-8), 5.91 (1H, s, H-4), 4.25 (3H, s, H-22), 4.10 (1H, d, J = 7.5 Hz, H-7), 2.18 (3H, s, H-23); ^{13}C NMR (10% $CD_3OD/CDCl_3$) δ 177.0 (qC, C-5), 176.5 (qC, C-3), 165.1 (qC, C-11), 154.1 (CH, C-1), 146.1 (qC, C-19), 143.1 (CH, C-17), 139.0 (qC, C-10), 130.7 (CH, C-14), 125.8 (CH, C-8), 123.9 (qC, C-15), 119.7 (qC, C-12), 119.1 (qC, C-21), 119.0 (qC, C-2), 114.5 (CH, C-4), 113.1 (CH, C-16), 109.3 (qC, C-20), 103.5 (CH, C-7), 50.2 (qC, C-6), 37.4 (CH_3, C-22), 14.8 (CH_3, C-23); HREIMS m/z 438.9996, 440.9869 $[M^+]$ (calcd for $C_{20}H_{14}{}^{79}BrN_3O_2S$, 438.9990 and $C_{20}H_{14}{}^{81}BrN_3O_2S$, 440.9863).

Discorhabdin U (3): UV (MeOH) λ_{max} 425 (log ϵ 3.12), 340 (3.95), 287 (4.01), 242 (4.22), 205 (4.42) nm; IR (NaCl, neat) ν_{max} 3328, 2924, 1637, 1602, 1558, 1479, 1435, 1280, 1010, and 668 cm^{-1}; ^1H NMR ($CDCl_3$) δ 7.57 (1H, s, H-1), 6.99 (1H, br s, H-9), 6.63 (1H, s, H-14), 6.38 (1H, d, J = 7.5 Hz, H-8), 5.88 (1H, s, H-4), 4.09 (1H, d, J = 7.5 Hz, H-7), 3.93 (2H, t, J = 8.0 Hz, H-17), 3.90 (3H, s, H-22), 2.58 (2H, t, J = 8.0 Hz, H-16), 2.29 (3H, s, H-23); ^{13}C NMR ($CDCl_3$) δ 176.6 (qC, C-5), 175.2 (qC, C-3), 169.4 (qC, C-11), 154.2 (CH, C-1), 154.0 (qC, C-19), 137.1 (qC, C-10), 128.4 (CH, C-14), 125.6 (CH, C-8), 122.3 (qC, C-21), 122.0 (qC, C-12), 119.0 (qC, C-2), 117.2 (qC, C-15), 114.6 (CH, C-4), 109.0 (qC, C-20), 104.6 (CH, C-7), 50.3 (CH_2, C-17), 49.7 (qC, C-6), 35.4 (CH_3, C-22), 17.9 (CH_2, C-16), 15.1 (CH_3, C-23); HRLSIMS (3-nitrobenzyl alcohol) m/z at 442.0275, 444.0052 $[M + H]^+$ (calcd for $C_{20}H_{17}{}^{79}BrN_3O_2S$, 442.0225, $C_{20}H_{17}{}^{81}BrN_3O_2S$, 444.0470).

Biological Activity. Compounds **1**, **2**, and **3** exhibited in vitro cytotoxicity against cultured murine P-388 tumor cells, with IC_{50} values of 3.08, >5, and 0.17 μM, respectively. Cytotoxicity was also observed for A-549 human lung adenocarcinoma cells, with IC_{50} values of >5, >5, and 0.17 μM and for PANC-1 human pancreatic cells with IC_{50} values of 2.6, 0.7, and 0.069 μM, respectively.[16]

P-388, A-549, and PANC-1 Cytotoxicity Assays. The compounds were analyzed as to their effects on proliferation of A549 human adenocarcinoma, PANC-1 human pancreatic, and P-388 murine leukemia cell lines. P-388 cells were obtained from Dr. R. Camalier, National Cancer Institute, Bethesda, MD, and A549 and PANC-1 cells were obtained from American Type Culture Collection, Rockville, MD. All cell lines were maintained in Roswell Park Memorial Institute (RPMI) medium 1640 supplemented with 10% fetal bovine serum. All cell lines are cultured in plastic tissue culture flasks and kept in an incubator at 37 °C in humidified air containing 5% CO_2. Prior to testing, antibiotic-free stock cultures of each of the cell lines were subcultured to 10^6 cells/mL by dilution in fresh growth medium at 2 to 3 day intervals. To assess the antiproliferative effects of compounds against the P-388 cell line, 200 μL cultures (96-well tissue culture plates, Nunc, Denmark) are established at 1×10^5 cells/mL in drug-free medium or medium containing the test agent at 10.0, 1.0, 0.10, and 0.010 μg/mL. Solvent for all dilutions is ethanol. All experimental cultures are initiated in medium containing gentamycin sulfate (50 μg/mL; Schering Corporation, Kenilworth, NJ). After 48 h exposures, P-388 cells are enumerated using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT).[17] Similar procedures are utilized for A-549 and PANC-1 cells, which require an additional 48 h exposure prior to MTT addition. Results are expressed as percent inhibition compared to the negative (no drug) control. Positive drug controls are included to monitor drug sensitivity of each of the cell lines. These include varying dilutions of 5-fluorouracil and adriamycin.

To quantitate the effects of pure compounds on cell proliferation and resulting IC_{50} values, 75 μL of warm growth media containing 5 mg/mL MTT is added to each well, and cultures are returned to the incubator and left undisturbed for 90 min. To spectrophotometrically quantitate formation of reduced formazan, plates are centrifuged (900*g*, 5 min), culture fluids are removed by aspiration, and 200 μL of acidified 2-propanol (2 mL of concentrated HCl/liter 2-propanol) is added per well. The absorbance of the resulting solutions is measured at 570 nm with a plate reader (TECAN Spectra II Plate Reader, TECAN U.S., Research Triangle Park, NC). The absorbance of test wells is divided by the absorbance of drug-free wells, and the concentration of agent that results in 50% of the absorbance of untreated cultures (IC_{50}) is determined by linear regression of logit-transformed data.[18] A linear relationship between tumor cell number and formazan production has been routinely observed over the range of cell densities observed in these experiments. The two standard drug controls (indicated above) are included in each assay as a check to monitor the drug sensitivity of each of the cell lines, and IC_{50} values are determined for each drug−cell combination.

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Supporting Information Available: A color photograph of the sponge sample *Batzella* sp. This material is available free of charge via the Internet at http://pubs.acs.org.

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