Petrobactin Sulfonate, a New Siderophore Produced by the Marine Bacterium *Marinobacter hydrocarbonoclasticus*

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Received May 25, 2004

Culture of the oil-degrading marine bacterium *Marinobacter hydrocarbonoclasticus* gave the known siderophore petrobactin (**1**) and the new metabolite petrobactin sulfonate (**2**), the first marine siderophore containing a sulfonated 3,4-dihydroxy aromatic ring. The structure of petrobactin sulfonate was elucidated from spectral data, resulting in a revision of the NMR assignments of petrobactin.

Crude oil is one of the most significant organic pollutants to the marine environment. Many compounds in crude oil are biodegradable, and this observation has fueled investigations on oil-degrading marine microorganisms. *Marinobacter hydrocarbonoclasticus* is a ubiquitous marine bacterium,[1,2] which grows on a variety of hydrocarbons as its sole carbon source. Iron is essential to the growth of the vast majority of microorganisms,[3] yet iron is present at very low concentrations (0.02−1 nM) throughout much of the world's surface ocean waters.[4−6] These low levels of iron have been shown to limit growth of many microorganisms.[4] In response to low iron environments, aerobic bacteria often produce siderophores, which are high-affinity iron(III)-binding compounds that facilitate Fe(III) transport into the bacterium.[7] While the structures of hundreds of terrestrial siderophores are known, relatively few structures of siderophores produced by marine bacteria have been elucidated.[5] A number of siderophores contain the 2,3-dihydroxybenzoate moiety, which functions in Fe(III) coordination; however, until the recent discovery of petrobactin (**1**),[8,9] no example with 3,4-dihydroxy substitution had been published. In contrast to a plethora of reported aliphatic sulfonates,[10] sulfonation of the aromatic groups is rather rare in the natural product literature. Interestingly, the known sulfonated aromatic natural products are siderophores derived from either terrestrial *Pseudomonad*[11,12] or marine *Pseudoalteromonad*[13] species. We report herein the structural characterization of a new siderophore, petrobactin sulfonate (**2**), isolated from the oil-degrading marine bacterium *Marinobacter hydrocarbonoclasticus*.

The siderophores produced in the culture of *M. hydrocarbonoclasticus* were isolated by adsorption to Amberlite XAD-2 resin after removing the bacterial cells by centrifugation and acidifying the culture medium to pH 2.5−3. The siderophores were eluted with methanol. Preparative-scale reversed-phase HPLC of this methanol fraction resulted in the isolation of the known petrobactin (**1**)[8,9] and the new compound petrobactin sulfonate (**2**), with typical yields of 1.2 and 0.55 mg per liter of culture medium, respectively.

High-resolution electrospray mass spectrometry (*m/z* 799.3199 $[M + H]^+$, $\Delta = +1.5$ mmu), in combination with ^{1}H and ^{13}C NMR data (Table 1), gave the molecular formula $C_{34}H_{50}N_6O_{14}S$ (13 double-bond equivalents). The presence of sulfur was confirmed by elemental analysis. More significantly, a mass difference of 80 Da from the $[M + H]^+$ molecular ion of petrobactin (HRFABMS *m/z* 719.3614)[8] suggested the addition of a sulfonate group. Further evidence supporting the presence of a sulfonate group was obtained from tandem ESI-MS in negative ion mode, considering the species at *m/z* 797.2. At a collision voltage of >150 V, a peak of *m/z* 79.9, attributable to a sulfonate moiety, was observed.

The ^{1}H and ^{13}C NMR assignments for petrobactin sulfonate (**2**) were confirmed by gCOSY, HSQC, gHMBC, and CIGAR[14] 2D experiments (Table 1). The splitting patterns observed in the aromatic region of the ^{1}H NMR spectrum were indicative of the unusual 3,4-dihydroxybenzoyl moiety, a functionality unique to petrobactin (**1**) and petrobactin sulfonate (**2**) in the marine siderophore literature. The presence of a single correlation for one of the aromatic rings in the gCOSY experiment, from H-5 (δ 6.76) to H-6 (δ 7.18), also supported 3,4-disubstitution. It was not possible to see the equivalent correlation from the other aromatic ring in this experiment, due to the closeness in chemical shift of the H-5′ (δ 6.76) and H-6′ (δ 6.72) protons. All correlations observed in the gHMBC and CIGAR experiments in the aromatic region were consistent with the presence of two catechol rings, including two $^4J_{CH}$ correlations, from H-6 (δ 7.18) to C-3 (δ 144.9) and H-6′ (δ 6.72) to C-3′ (δ 142.3), in the CIGAR experiment. No evidence to differentiate the hydroxyl protons at the 3, 4, and 4′ positions was obtained (δ 9.12, 9.16, and 9.54, respectively); however, the 3′-OH proton was able to be assigned at 11.23 ppm, due to observed correlations in the gHMBC and CIGAR experiments to C-2′ (δ 127.0), C-3′ (δ 142.3), and C-4′ (δ 147.2).

The aromatic splitting patterns in the ^{1}H NMR spectrum also indicated that the sulfonate group was attached to one of the catechol rings of the previously symmetrical petrobactin molecule. The H-2 doublet (δ 7.27), H-5 doublet (δ 6.76), and H-6 doublet of doublets (δ 7.18) remained from the ^{1}H NMR spectrum of petrobactin; however these resonances integrated as one proton, two protons, and one proton, respectively. Thus, the doublet at 6.76 ppm accounted for both H-5 and H-5′. The doublet of doublets at 7.18 ppm, assigned to H-6 and due to the combination of H-5 vicinal (ortho) coupling (8.5 Hz) and H-2 meta coupling (2 Hz), was not seen for H-6′. Instead, a one-proton doublet,

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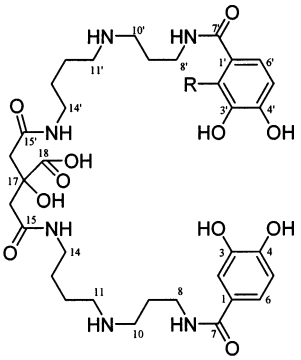
10.1021/np049823i CCC: $27.50
Published on Web 10/08/2004

Table 1. ^{1}H, ^{13}C, and 2D NMR Data for Petrobactin Sulfonate (**2**)

position	δ_H mult (*J* in Hz)	δ_C	gCOSY	HSQC	gHMBC and CIGAR[a]
1	na	125.4			
1′	na	126.6			
2	7.27 d (2)	115.0		C-2	C-1 (wk), C-3, C-4, C-6, C-7
2′	na	127.0			
3	na	144.9			
3′	na	142.3			
4	na	148.5			
4′	na	147.2			
5	6.76 d (8.5)	114.8	H-6	C-5	C-1, C-2 (wk),[b] C-3, C-4, C-7
5′	6.76 d (8.5)	115.5		C-5′	C-1′, C-3′, C-4′, C-7′ [b]
6	7.18 dd (2, 8.5)	119.0	H-5	C-6	C-2, C-3 (wk),[b] C-4 (wk),[b] C-7
6′	6.72 d (8.5)	119.6		C-6′	C-2′, C-3′, C-4′, C-7′
7	na	166.6			
7′	na	169.3			
8/8′	3.27 m	36.1, 36.2	H-9/H-9′, N-1H/N-1′H	C-8/C-8′	C-7/C-7′, C-9/C-9′, C-10/C-10′
9	1.80 m	26.3	H-8, H-10	C-9	C-8, C-10
9′	1.80 m	25.6	H-8′, H-10′	C-9′	C-8′, C-10′
10	2.89 m	44.8	H-9, N-2H	C-10	C-8 (wk)[b]
10′	3.12 m	44.4	H-9′, N-2′H	C-10′	C-8′,[c] C-9′[c]
11/11′	2.89 m	46.5	H-12/H-12′, N-2H/N-2′H	C-11/C-11′	C-13/C-13′ (wk)[b]
12/12′	1.56 m	23.0	H-11/H-11′, H-13/H-13′	C-12/C-12′	C-11/C-11′,[c] C-13/C-13′,[c] C-14 /C-14′ (wk)
13/13′	1.43 m	26.0	H-12/H-12′, H-14/H-14′	C-13/C-13′	C-11/C-11′, C-12 /C-12′,[c] C-14 /C-14′
14/14′	3.04 m	37.7	H-13/H-13′, N-3H/N-3′H	C-14/C-14′	C-12/C-12′, C-13 /C-13′, C-15 /C-15′
15/15′	na	169.5			
16/16′	2.50 d (15) 2.58 dd (4, 15)	43.3		C-16/C-16′	C-15 /C-15′, C-16′ /C-16, C-17, C-18
17	na	73.5			
18	na	175.0			
N-1H	8.31 t (6)	na	H-8		C-7, C-8
N-1′H	8.19 t (6)	na	H-8′		C-7′, C-8′
N-2H	8.27 m	na	H-10, H-11		
N-2′H	8.23 m	na	H-10′, H-11′		
N-3H/N-3′H	7.99 t (5)	na	H-14/H-14′		C-14 /C-14′, C-15 /C-15′
OH (C-3′)	11.23 s	na			C-2′, C-3′, C-4′
OH (C-3/C-4 /C-4′)	9.12, 9.16, 9.54 (all br s)	na			

[a] gHMBC and CIGAR experiments run with coupling constants optimized at 5 and 5−10 Hz, respectively. [b] Correlation only seen in the CIGAR experiment. [c] Weak correlation observed in the CIGAR experiment.



1: R = H
2: R = SO_3H

with H-5′ ortho coupling only (8.5 Hz), was observed for this proton (δ 6.72). The lack of meta coupling in this signal indicated substitution at the 2′ position. In addition, no signal assignable to a proton attached to C-2′ was observed. Thus, it was inferred that the sulfonate was in the 2′ position.

The connectivity of the molecule was determined via gCOSY, gHMBC, and CIGAR correlations (Table 1). The gCOSY experiment unequivocally established the connectivity of the two spermidinyl moieties in the molecule. In particular, gCOSY correlations from H-11 (δ 2.89) and H-13 (δ 1.43) to H-12 (δ 1.56) and from H-12 and H-14 (δ 3.04) to H-13 (along with the equivalent correlations to H-12′ and H-13′) required a reversal of the ^{1}H and ^{13}C assignments at the 12 and 13 positions from those previously reported for petrobactin (**1**).[8,9] To confirm that such a reversal should also apply in the case of petrobactin itself, a gCOSY experiment was run on a petrobactin sample. In this experiment, correlations from H-11 (δ 2.89) and H-13 (δ 1.42) to H-12 (δ 1.55) and from H-12 and H-14 (δ 3.04) to H13 confirmed that the previously reported ^{1}H and ^{13}C assignments at positions 12 and 13 in this molecule did in fact need to be interchanged.

The attachment of each catechol ring to a spermidinyl group via a carbonyl group on both sides of the molecule was established through gHMBC and CIGAR correlations (see Table 1), including two $^{4}J_{CH}$ correlations, from H-5 (δ 6.76) to C-7 (δ 166.6) and H-5′ (δ 6.76) to C-7′ (δ 169.3), in the CIGAR experiment. These experiments also established the connectivity of the N3/N3′ ends of the spermidinyl groups to a single citryl moiety, as well as the connectivity of the citryl group itself.

It appears that the structure of petrobactin sulfonate (**2**) is in the form of a double zwitterion involving N-2 and N-2′ and the sulfonate and carboxylate moieties. The strongest evidence for this lies in the observed chemical shifts for the N-2 and N-2′ protons (δ 8.27 and 8.23, respectively), which are in the correct range for protons on a positively charged nitrogen in an alkyl chain (~6−9 ppm) and significantly downfield from the expected chemical shift for protons on a neutral nitrogen atom in an alkyl chain (0.5−4 ppm).[15] Further evidence is to be found in the observed proton and carbon chemical shifts at the adjacent 10/10′

and 11/11′ positions (Table 1),[15,16] in the integrals of the protons attached to nitrogen in the ^{1}H NMR spectrum, and in the splitting of H-10′ in the same spectrum (triplet of triplets, appearing as a "quintet").

In conclusion, we report a new sulfonated siderophore, petrobactin sulfonate (**2**). Interestingly, only one of the two catecholate groups of the otherwise symmetrical molecule was sulfonated; thus C-17 is a stereogenic center in petrobactin sulfonate ($[\alpha]^{20}_D$ −2.5° (*c* 0.013, DMSO)), but not in petrobactin. A possible functional significance in the outer membrane recognition process has not yet been investigated. The sulfonate functionality was unequivocally established to be vicinal to the two hydroxyl groups on one of the catecholate ring systems, as is the case for the sulfonated dihydropyoverdins.[12] The sulfonated form of petrobactin is more hydrophilic than petrobactin, which results in shorter retention times in RP-HPLC and possibly also in reduced membrane permeability. The altered physicochemical properties might also have a function in the particular environment of *M. hydrocarbonoclasticus* at the interface of seawater to oil hydrocarbons. In an analogous case, TRENCAM, a synthetic siderophore analogue containing the aromatic 2,3-dihydroxy catechol group, was rendered more water-soluble by sulfonation.[17] Future studies of the metabolism of petrobactin sulfonate may provide new insight into the biosynthetic and biodegradative pathways of aromatic sulfonates.

Experimental Section

General Experimental Procedures. The optical rotation was measured at 589 nm (Na_D) using a Perkin-Elmer 341 polarimeter. The UV spectrum was measured on a Cary 300 spectrophotometer. ^{1}H, ^{13}C, and 2D NMR (^{1}H−^{1}H gCOSY, ^{1}H−^{13}C HSQC, ^{1}H−^{13}C gHMBC, and ^{1}H−^{13}C CIGAR) spectra were recorded on a Varian INOVA 500 MHz spectrometer. Electrospray-ionization mass spectra (ESI-MS) were recorded in both the positive and negative modes on a Micromass (Manchester, UK) quadrupole time-of-flight (QTOF-2) mass spectrometer. The elemental analysis was carried out by Quantitative Technologies Inc. (QTI). HPLC grade solvents and doubly deionized Nanopure water were used throughout.

Culture and Isolation. *M. hydrocarbonoclasticus* (SP.17; ATCC 49840)[18,19] was cultured in a hypersaline medium (0.5 M/29.22 g NaCl, 0.4 g $MgSO_4 \cdot 7H_2O$, 1.0 g NH_4Cl, 3.0 g K_2HPO_4, 0.15 g $CaCl_2 \cdot 2H_2O$, and 5.0 g sodium succinate/$Na_2C_4H_4O_4 \cdot 6H_2O$ per liter of Nanopure water) in order to optimize siderophore production. The cultures were typically grown as 2 L cultures in 4 L flasks on a rotary shaker (150 rpm) for 7 days. At the time of harvesting, cultures were in stationary phase. After centrifugation of the culture medium (5000 rpm, 20 min), Amberlite XAD-2 resin (Supelco) was added to the decanted supernatant (ca. 100 g/L), and the resultant mixture was shaken for at least 4 h at >100 rpm. The XAD was then loaded into a glass chromatography column (2 cm internal diameter), washed with one column volume of Nanopure water, and eluted with 100% methanol. Using the solution-phase Chrome Azurol S (CAS) assay with shuttle,[20] the methanol fraction was found to contain the siderophores. Since the culture medium remained CAS-positive, it was acidified to pH 2.5, re-extracted with XAD resin, and shaken at >100 rpm for at least 6 h. The XAD was again recovered, washed, and eluted as described above. The final purification was achieved on the combined methanol fractions using preparative reversed-phase HPLC (Vydac C4 column (10 μm, 22 mm i.d. × 250 mm) using a CH_3CN/H_2O gradient (0% to 60%) over 35 min, with 0.1% trifluoroacetic acid). Acid was removed from the samples via repeated addition and evaporation of methanol during the drying process.

Petrobactin sulfonate (2): white solid; $[\alpha]^{20}_D$ −2.5° (*c* 0.013, DMSO); UV (DMSO) λ_{max} (log ϵ) 229 (3.95), 253 (3.83), 291 (3.74) nm; ^{1}H NMR (DMSO-d_6, 500 MHz) see Table 1; ^{13}C NMR (DMSO-d_6, 125 MHz), see Table 1; ESI-MS *m/z* 799.3199 $[M + H]^+$ (calcd for $C_{34}H_{51}N_6O_{14}S$, 799.3184).

Acknowledgment. Funding from NIH GM38130 and The Center for Environmental BioInorganic Chemistry (CEBIC), an NSF Environmental Molecular Science Institute (CHE-0221978), is gratefully acknowledged. We thank J. Pavlovich (mass spectrometry) and A. Shirazi (NMR) for technical assistance, and A. M. Cook (Department of Biology, University of Konstanz) for useful discussions.

Supporting Information Available: ^{1}H NMR, ^{13}C NMR, gCOSY, HSQC, gHMBC, and CIGAR spectra of petrobactin sulfonate. ^{1}H NMR and gCOSY spectra of petrobactin. This material is available free of charge via the Internet at http://pubs.acs.org.

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NP049823I