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Motualevic Acids A−F, Antimicrobial Acids from the Sponge *Siliquariaspongia* sp.

Jessica L. Keffer, Alberto Plaza, and Carole A. Bewley*

Laboratory of Bioorganic Chemistry, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland 20892

caroleb@mail.nih.gov

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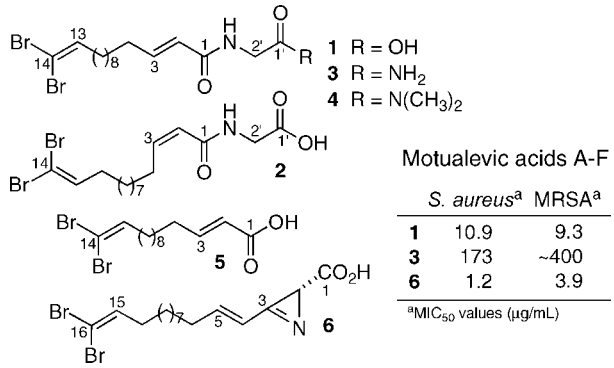
ABSTRACT



Seven new antibacterials, motualevic acids A−F (1−6) and (4*E*)-(*R*)-antazirine (7), have been isolated from the marine sponge *Siliquariaspongia* sp. and their structures elucidated by spectroscopic methods. Motualevic acids A−D are the first glycyl conjugates of the ω-brominated lipid (*E*)-14,14-dibromotetradeca-2,13-dienoic acid, and motualevic acid F is the first long-chain 2*H*-azirine 2-carboxylic acid to be found in nature. Carboxylic acid-containing compounds 1 and 6 inhibit the growth of *Staphylococcus aureus* and methicillin-resistant *S. aureus* at 1.2−10.9 μg/mL.

The steady increase in numbers of drug-resistant bacterial infections is a pressing issue in global health. Decades of antibiotic use combined with the multiple challenges of and limited efforts toward the antibiotic drug discovery process have in part led to the existence of multiple groups of drug-resistant microorganisms.[1,2] Prevalent examples include methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant Enterococci, and multidrug-resistant tuberculosis (MDR-TB). Programs tackling discovery and development of new antibiotics continue to be necessary, and the value of natural products in such efforts is by now well appreciated.[2−4]

Recently, we have described a number of new cyclic peptides obtained from lithistid sponge collections, each of which exhibits antimicrobial, antifungal, or HIV-inhibitory activity. Examples include mirabamides A−D[5] that strongly inhibit growth of *Bacillus subtilis*, *Candida albicans*, and HIV-1 envelope-mediated membrane fusion (or HIV-1 entry) and lipophilic peptide lactones celebesides A−C[6] that also inhibit HIV-1 entry. Although studied for many years and by several groups, lithistid demosponges continue to be extraordinarily rich sources of new marine natural products, many of which feature hallmarks of prokaryotic biosynthetic

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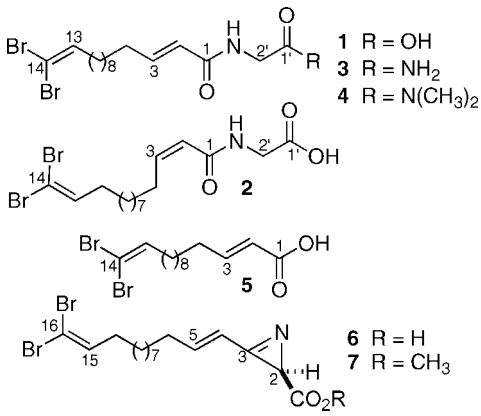
origins.[7] Continuing with our efforts to identify new anti-infectives from marine organisms, a Fijian specimen of *Siliquariaspongia* sp. whose crude extracts strongly inhibited the growth of *Staphylococcus aureus* and MRSA was investigated. Antimicrobial assay- and LC−MS-guided fractionation led to the isolation of six new brominated long-chain acids termed motualevic acids A−F (**1**−**6**), along with a new enantiomer of antazirine (**7**). The combinatorial nature of the suite of natural products present in this sponge provides a clear structure−activity relationship for MRSA activity. The details of the isolation, structure elucidation, and antimicrobial activities of motualevic acids are described here.



The marine sponge *Siliquariaspongia* sp. was collected around Motualevu reef in Fiji at a depth of −40 m.[8] The freeze-dried sponge was extracted sequentially with H_2O and MeOH/CH_2Cl_2 (1:1) to provide crude aqueous and organic extracts, both of which inhibited the growth of *S. aureus* and MRSA in a disk diffusion assay. The *n*-butanol-soluble material from the aqueous extract and $CHCl_3$-soluble material from the organic extract were chromatographed on Sephadex LH-20 eluting with 3:1 MeOH/H_2O to provide several active fractions that were combined and further purified by reversed-phase HPLC (65−85% MeOH in 0.05% TFA) to give pure compounds **1**−**7**.

Motualevic acid **1** was obtained as a colorless solid (18.6 mg) that gave an $[M - H]^-$ ion at *m*/*z* 436.0126 (calcd for $C_{16}H_{24}Br_2NO_3$, 436.0123). The 1:2:1 isotopic distribution at *m*/*z* 436, 438, and 440 secured the presence of two Br atoms and was consistent with the molecular formula $C_{16}H_{25}Br_2NO_3$, requiring four sites of unsaturation. The downfield region of the ^{1}H NMR spectrum (Table 1) contained signals for three olefinic protons at δ 6.84 (1H, dt, J = 15.3, 7.3 Hz), 6.50 (1H, t, J = 7.3 Hz), and 6.00 (1H, d, J = 15.3 Hz) and a two-proton singlet at δ 3.98 (2H, s). The presence of two methylene signals at δ 2.13 (2H, dd, J = 7.3, 14.6) and 2.24 (2H, dd, J = 7.3, 14.4) and a methylene envelope from δ 1.35−1.50 (14H) in the upfield

Table 1. NMR Data for **1** (CD_3OD)

	δ_C[a]	δ_H[b] (J in Hz)	HMBC[c]
1	168.6		
2	124.4	6.00 d (15.3)	C1, C3 C4, C5
3	146.6	6.84 dt (15.3, 7.3)	C1, C2, C4, C5
4	33.2	2.24 dd (7.3, 14.4)	C1, C2, C3, C5, C6
5	29.6	1.50 m	C3, C4, C6
6	30.2	1.35	
7	30.5	1.35	
8	30.6	1.35	
9	30.7	1.35	
10	30.4	1.35	
11	28.9	1.47 m	C10, C12, C13
12	34.1	2.13 dd (7.3, 14.6)	C10, C11, C13, C14
13	140.5	6.50 t (7.3)	C11, C12, C14
14	88.6		
1′	172.7		
2′	41.9	3.98 s	C1, C1′, C2

[a] Recorded at 125 MHz, referenced to residual CD_3OD at 49.15 ppm. [b] Recorded at 500 MHz, referenced to residual CD_3OD at 3.33 ppm. [c] HMBC correlations from indicated proton, $^{2,3}J_{C-H}$ = 6 Hz.

portion of the spectrum indicated compound **1** contained an unsaturated fatty acid. Similarly, the ^{13}C NMR spectrum (Table 1) contained three olefinic carbons at δ 124.4, 146.6, and 140.5, nine methylenes at δ 28.9−34.1, a carbonyl at δ 168.6, and a quaternary carbon at δ 88.6 characteristic of a terminal dibromovinyl moiety.[9,10] COSY and TOCSY correlations revealed spin systems corresponding to C-2 to C-5 and C-11 to C-13, both of which were correlated to the methylenes at δ 1.35; while the methylene at δ 3.98 (2H, s) represented a separate spin system. HMBC correlations (Figure 1) from the olefinic proton at δ 6.50 to the

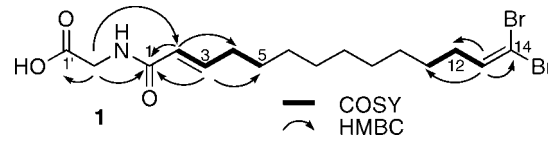


Figure 1. COSY and selected HMBC correlations observed for motualevic acid A (**1**).

dibromovinyl carbon at δ 88.6, and the olefinic protons at δ 6.84 and 6.00 to the carbonyl at δ 168.6 established the structure of the 14-carbon chain. The requirement of a nitrogen atom together with the HMBC correlations from the methylene singlet at δ 3.98 to the carbonyls at δ 168.6 (C-1) and 172.7 (C-1′) revealed the presence of a glycine residue coupled to the acid to give compound **1**. The *E* geometry of the double bond at C-2/C-3 was apparent from the large $^2J_{H2-H3}$ value of 15.3 Hz. To our knowledge, this

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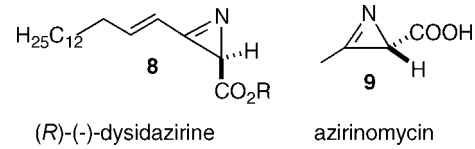
is the first example of a glycyl lipid occurring in a natural product.

Motualevic acid B (**2**), one of the five minor components in the extract, was shown by HR-ESIMS to have the same molecular weight and formula as **1**. The major differences in the ^{1}H NMR spectra of **1** and **2** were found in the chemical shifts and coupling constants of the olefinic protons at δ 5.88 (1H, d, $J = 11.6$ Hz) and 6.05 (1H, dt, $J = 11.6$ and 7.5 Hz) indicating compound **2** to be the *Z* isomer of **1**. On the basis of HR-ESIMS, the molecular formulas of motualevic acids C (**3**) and D (**4**) were established as $C_{16}H_{25}Br_2N_2O_2$ (*m*/*z* 437.0446 $[M + H]^+$) and $C_{18}H_{30}Br_2N_2O_2$ (*m*/*z* 465.0746 $[M + H]^+$), respectively, suggesting replacement of the −OH by a primary amine in **3** and by *N*,*N*-dimethylamine in **4**. Consistent with the mass spectral data, the only changes observed in the ^{1}H NMR spectra were traced to the glycine moiety. In particular, the ^{1}H NMR spectrum of **3** in $CDCl_3$ showed the presence of an amide signal at δ 6.08 (2H), while the spectrum of **4** (CD_3OD) showed the presence of two *N*-methyls at δ 2.98 and 3.08, both of which showed HMBC correlations to C-1′ at δ 170.2. Thus, **3** and **4** are the glycinamide and *N*,*N*-dimethylglycinamide derivatives of **1**. Both glycinamide and *N*,*N*-dimethylglycinamide are new residues in natural products.

Motualevic acid E (**5**), present in the aqueous extract only, was shown by HR-ESIMS to have the molecular formula $C_{14}H_{22}Br_2O_2$ (*m*/*z* 378.9908), indicating a loss of C_2H_3NO relative to motualevic acid A. Similarly, ^{1}H and ^{13}C NMR spectra for **5** were nearly identical to those of **1**, except for the absence of signals corresponding to the glycine residue. Thus, the structure of **5** must be the free (*E*)-14,14-dibromotetradeca-2,13-dienoic acid unit.

Two additional compounds **6** and **7** were also found exclusively in the aqueous extract. The HR-ESIMS of **6** again showed the characteristic isotopic pattern of two bromines, at *m*/*z* 418.0009, 419.9980, and 421.9957 $[M - H]^-$, giving a molecular formula $C_{16}H_{23}Br_2NO_2$. Similarities between the NMR data of **1** and **6** suggested the presence of the same ω-brominated tetradecadienoic acid present in **1**−**5**; however, the glycine signals were conspicuously absent. Instead, a proton signal at δ 2.55 (1H, s), showing an HMBC correlation to the quaternary carbon at δ 156.1, along with a band in the FT-IR spectrum at ν 1770 cm^{-1}, suggested the presence of an azirine ring.[10,11] On the basis of 1D and 2D NMR data (Supporting Information), the structure of **6**, or motualevic acid F, was established as (*E*)-3-(13,13-dibromotrideca-1,12-dienyl)-2*H*-azirine-2-carboxylic acid. In addition, azirine **6** was strongly levorotatory ($[\alpha]_D$ −75.0), indicating a 2*R* configuration.[10,12] Finally, spectroscopic data showed compound **7** to be the methyl ester of azirine **6**, and an $[\alpha]_D$ of −7.3 indicated a 2*R* configuration, confirming **7** to be (4*E*)-*R*-antazirine, a new enantiomer of the antazirine series.

Long-chain azirine-2-carboxy methyl esters, exemplified by **7** and the nonhalogenated lipid (4*E*)-(*R*)-dysidazirine[11]



(*R*)-(-)-dysidazirine azirinomycin

Figure 2. (*R*)-(−)-Dysidazirine (**8**) isolated from the marine sponge *Dysidea fragilis*[11] and azirinomycin (**9**) isolated from *Streptomyces aureus*.[15]

(**8**, Figure 2), have been isolated previously from collections of the marine sponge *Dysidea fragilis* and are known to exist as mixtures of enantiomers.[9−11] Using chiral HPLC and polarimetry, Skepper and Molinski recently demonstrated that dysidazirines and antazirines can racemize spontaneously and that their (4*Z*)-isomers occur with higher optical purity than the corresponding (*E*)-isomers.[10] In our case, chiral HPLC[13] showed an 80% enantiomeric excess for (4*E*)-2*H*-azirine 2-carboxylic acid **6**, significantly higher than optical purities reported for other (4*E*) isomers, while the optical rotation of methyl ester **7** indicates lower optical purity. These measurements suggest factors in addition to configuration of the C-4/C-5 olefin contribute to rate of racemization.

Antimicrobial disk diffusion assays performed with pure motualevic acids A−F (**1**−**6**) and (4*E*)-(*R*)-antazirine (**7**) traced the MRSA-inhibitory activity to acids **1** and **6**, which inhibited the growth of MRSA at loadings of 10 and 5 μg/disk, respectively (Table 2). The same assay performed with

Table 2. Antimicrobial Screening Results for **1**−**7**

	agar disk diffusion[a]		microbroth dilution assay[b]	
compd	*S. aureus*	MRSA	*S. aureus*	MRSA
1	10	10	10.9± 4.0	9.3 ± 2.7
2	10	NA	–	–
3	NA	NA	173 ± 33	400 ± 110
4	NA	NA	–	–
5	50	NA	–	–
6	2	5	1.2 ± 0.3	3.9 ± 1.0
7	NA	NA	–	–

[a] Values in μg/disk where values chosen gave rise to zones of inhibition of 8−11 mm; NA (not active at loads as high as 25 μg/disk for compounds **2**−**4** and 50 μg/disk for compounds **5** and **7**). [b] MIC_{50} values reported as μg/mL; –, not tested.

S. aureus (SA) showed compounds **1**, **2**, **5**, and **6** to be active at respective loadings of 10, 10, 50, and 2 μg/disk. The observations that compound **2** inhibits the growth of SA but not MRSA (at concentrations up to 25 μg/disk), that amides **3** and **4** were inactive toward both strains, and that tetradecadienoic acid **5** weakly inhibits the growth of SA suggest antimicrobial activity toward MRSA is dependent on the presence of a carboxylic acid (in the form of glycine or 2-carboxylate) and 4*E* geometry. Compounds for which

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(13) YMC chiral NEA column, 0.01% TFA in 70:30 hexanes/*i*-PrOH.

sufficient material was available were further evaluated in liquid culture[14] giving results similar to those of the disk diffusion assay, and for cytotoxicity toward a kidney cell line (see the Supporting Information). Compound **6** represents the first example of a long-chain 2*H*-azirine-2-carboxylic acid and the second example of a 2*H*-azirine-2-carboxylic acid, with the first being the 3-methylazirine azirinomycin[15] (**9**, Figure 2). It is interesting to note that compound **6** was the most potent antibacterial agent, inhibiting the growth of SA and MRSA at concentrations as low as 2 and 5 μg disk, while its methyl ester **7** was inactive at concentrations as high as 50 μg/disk, consistent with a lack of antibacterial activity reported for other antazirines, all of which contain methyl esters.[9,10] Similarly, azirinomycin (**9**) was reported to show broad spectrum antimicrobial activity in its naturally occurring acid form, while that of its synthetic methyl ester derivative was greatly diminished. Compounds **1**−**7** were not active against *Candida albicans*, *Escherichia coli*, *Bacillus subtilis*, *Pseudomonas aeruginosa*, or *Enterococci faecium*.

The unusual structure and rare occurrence of (2*H*)-azirines in Nature[9−11,15] has spurred hypotheses for azirine biosynthesis, an area in which experimental information is absent. Possessing the same 2*R* configuration and C_{18} chain length, Molinski and Ireland observed that dysidazirine may share elements of D-sphingosine biosynthesis.[11] Upon discovery of the brominated natural product antazirine,[9] Faulkner suggested azirine ring formation may involve bromination/dehydrobromination, while Lowden proposed that azirinomycin and dysidazirine are generated by cyclization of α-amino-β-keto carboxyl intermediates of threonine or sphingosine, respectively.[16] With their recent finding of the first chlorinated antazirines,[10] Molinski hypothesized that azirine biosynthesis proceeds through cryptic double halogenation and cyclization (Figure 3a), analogous to the biosynthesis of coronamic acid,[17] a cyclopropane-containing natural product. Although reasonable, the origin of the nitrogen atom in the last scheme remained in question. Here, we have found that 2-carboxy-2*H*-azirine **6** co-occurs with the glycine adduct of 14,14-dibromotetradeca-2,13-dienoic acid (**1**), providing a possible precursor to and nitrogen atom source for synthesis of azirine **6** (Figure 3b) proceeding through base-induced cyclization without the need for halogenation.

Here we have described six new antibacterial, ω-terminus dibrominated acids that incorporate glycine, glycinamide, or *N*,*N*-dimethylglycinamide or a rare 2-carboxy-2*H*-azirine

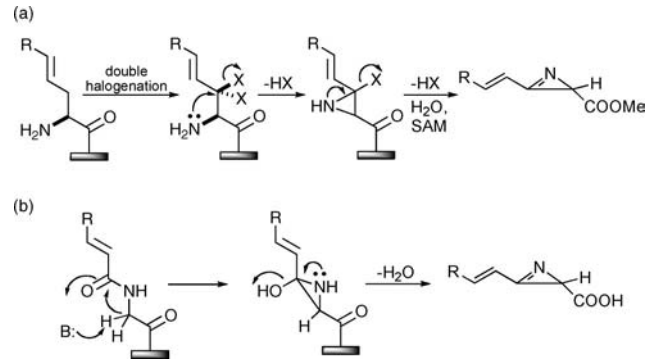

Figure 3. Proposed schemes for the biosynthesis of (a) 2*H*-azirine 2-carboxymethyl esters found in *Dysidea fragilis*, hypothesized to occur through cryptic halogenation[10,17] and (b) 2*H*-azirine 2-carboxylic acid found in *Siliquariaspongia* sp., starting from a glycyl lipid and proceeding through base-induced cyclization.

ring. While ω-dibrominated unsaturated fatty acids such as **5** have been isolated from several species of *Xestospongia*,[18] this is the first report of this type of compound occurring in a lithistid sponge. Likewise, prior to this report, long-chain azirines have been found exclusively in the marine sponge *Dysidea fragilis*, unrelated to either *Xestospongia* or *Siliquariaspongia*. Finally, motualevic acid F (**6**) provides the first example of a long-chain 2*H*-azirine containing a C-2 carboxylic acid and inhibits the growth of MRSA. Together with the antimicrobial results for glycyl lipid **1**, these studies suggest that the presence of a free acid is necessary for inhibition of MRSA in both compound types. The structures reported here provide a starting point for optimization of new MRSA-inhibitory antibiotics as well as a probable precursor in the biosynthesis of 2*H*-azirine 2-carboxylates.

Acknowledgment. We thank the Coral Reef Research Foundation, David Newman (NCI), and the country of Fiji for sample acquisition, John R. Lloyd (NIDDK) for HRMS measurements, and Michelle Kelly and the National Cancer Institute for sponge taxonomy. This work was supported in part by the NIH Intramural Research Program (NIDDK) and the Intramural AIDS Targeted Antiviral Program, Office of the Director, NIH (C.A.B.).

Supporting Information Available: Experimental details, ^{1}H and ^{13}C NMR assignments and 1D and 2D NMR spectra for **1**−**7**, and chiral HPLC chromatogram of **6**. This material is available free of charge via the Internet at http://pubs.acs.org.

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(14) Because compounds containing free acids exhibited the strongest antimicrobial activity in disk diffusion assays, assays were performed in liquid cultures to assure diffusion was not charge dependent.

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