# Cadiolides A and B, New Metabolites from an Ascidian of the Genus *Botryllus*

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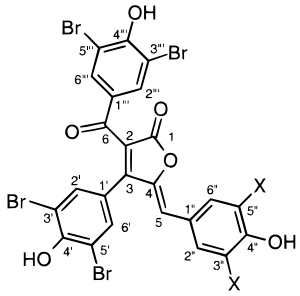
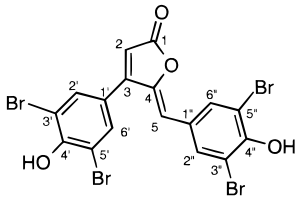
Marine ascidians are prolific producers of metabolites derived from amino acids.[1] Metabolites produced from phenylalanine or tyrosine are especially plentiful and include the tunichromes,[2] lamellarins,[3] the lukianols,[4] rigidin,[5] the polycitrons,[6] the ningalins[7] and the botryllamides.[8] The rubrolides,[9] isolated from *Ritterella rubra*, are examples of non-nitrogenous members of this group. We now wish to report the isolation of cadiolides A (**1**) and B (**2**) from an Indonesian *Botryllus* sp. The cadiolides do not contain nitrogen and are related in structure to the rubrolides, but possess a novel carbon skeleton.

Specimens of *Botryllus* sp. were collected by hand using scuba (−3 to −15 m) near Barrang Caddi in Indonesia and kept frozen until workup. Thawed samples of *Botryllus* sp. were homogenized and extracted with either EtOH or MeOH to give a crude extract. This extract was partitioned between hexane, $CHCl_3$, and aqueous MeOH. The aqueous MeOH soluble material was further fractionated by size-exclusion chromatography using Sephadex LH-20 (MeOH), resulting in the separation of a number of highly colored bands including yellow, orange, red, and black. Samples were inspected by TLC and $^1$H NMR spectroscopy and purified further by gradient silica flash chromatography (0−10% MeOH in $CHCl_3$) to give rubrolide A (**3**) and cadiolides A (**1**) and B (**2**) as amorphous solids.

Cadiolide A (**1**) was obtained as an orange amorphous solid that yielded an abundant ion cluster in both the negative ion ES-MS and MALDI-MS spectra centered at




cadiolide A (**1**) X=H
cadiolide B (**2**) X=Br




rubrolide A (**3**)

*m*/*z* 715 $(M - H)^-$. The positive ion MALDI-MS spectrum also showed an abundant ion cluster, this time centered at *m*/*z* 717 $(M + H)^+$. This isotopic packet corresponded to the presence of four bromine atoms (Figure 1, the top portion is the measured mass spectrum, and the bottom portion is the calculated mass spectrum for the proposed ion $C_{24}H_{13}Br_4O_6^+$). Note the excellent agreement in the relative abundances of the isotopic peaks. The most abundant ion was measured to be *m*/*z* 716.7398, which corresponds within 0.4 ppm to the most abundant calculated ion for $C_{24}H_{13}{}^{79}Br_2{}^{81}Br_2O_6^+$ at *m*/*z* 716.7401. This is consistent with the molecular formula $C_{24}H_{12}{}^{79}Br_2{}^{81}Br_2O_6$ possessing 17 degrees of unsaturation. The $^{13}$C NMR spectrum contained only 18 resonances, implying that there was some element(s) of symmetry present in a highly unsaturated aromatic structure. Only six proton resonances, all with chemical shifts greater that $\delta$ 6, were observed in the $^1$H NMR spectrum, and integration showed that four of the signals accounted for two protons each ($\delta$ 6.88, 7.55, 7.76, and 7.89), one for a single proton ($\delta$ 6.33) and one exchangeable signal accounted for three protons ($\delta$ 10.41). The UV spectrum showed absorptions at 254 and 406 nm, demonstrating the aromaticity and extended conjugation of **1**. The IR spectrum showed absorptions at 1721 and 1683 $cm^{-1}$. This, coupled with carbon resonances at $\delta$ 152.4, 157.4, 159.8, 165.5 and 185.0, indicated the presence of a number of aromatic hydroxyl groups and conjugated carbonyl groups.

The broad three-proton resonance at $\delta$ 10.26 in the $^1$H NMR spectrum was assigned to three exchangeable phenolic protons. An HMQC experiment established the proton and carbon one-bond connectivities in the structure. In particular, correlations were observed between carbons resonating at $\delta$ 116.2, 132.9, 133.5, and 133.7 and protons resonating at $\delta$ 6.88, 7.55, 7.76, and 7.89, respectively. This coupled with the observation of three-bond proton−carbon connectivities in an HMBC experiment between carbon resonances at $\delta$ 116.2, 132.9, 133.5, and 133.7 and proton resonances at $\delta$ 6.88, 7.55, 7.76, and 7.89, respectively, suggested the presence of a series

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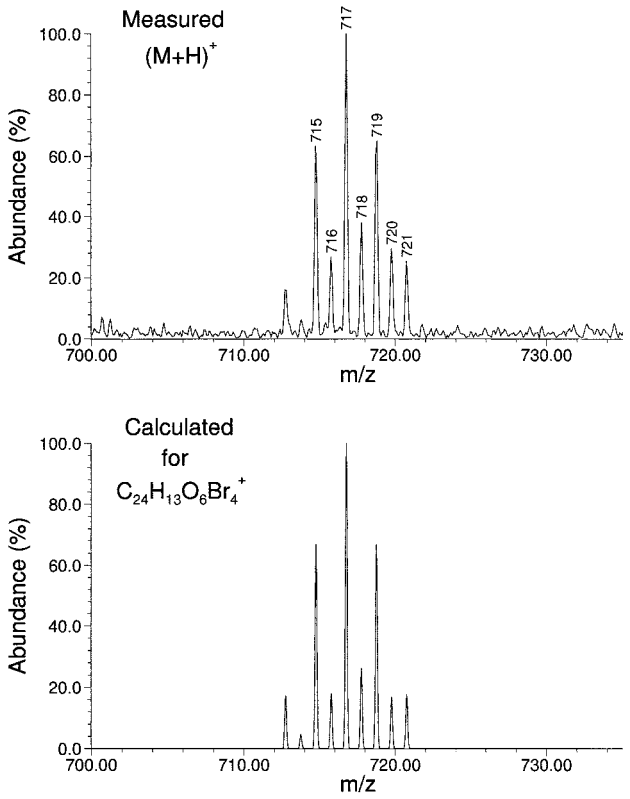


**Figure 1.** Comparison of the measured positive ion MALDI-MS spectrum of the molecular ion cluster to the theoretical spectrum of cadiolide A (**1**).

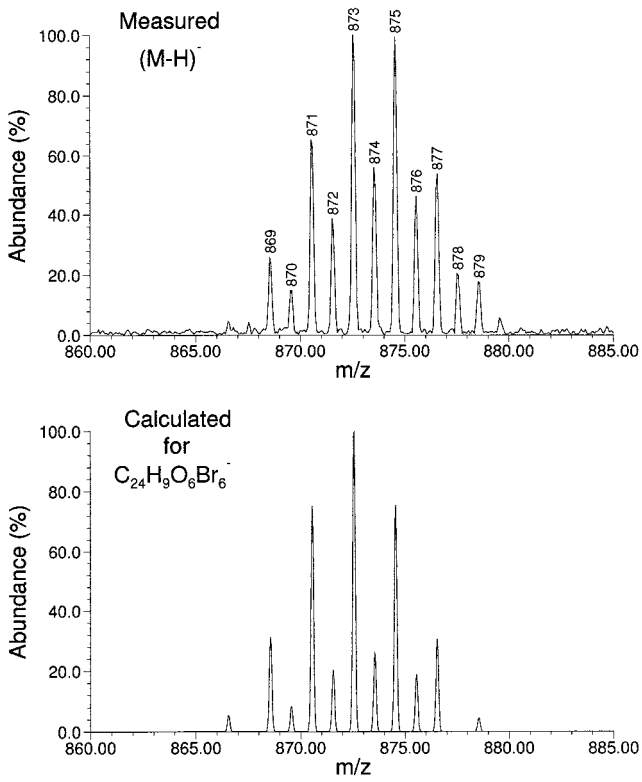


**Figure 2.** Comparison of the measured positive ion MALDI-MS spectrum of the molecular ion cluster to the theoretical spectrum of cadiolide B (**2**).

of four pairs of equivalent methine carbons situated meta to one another on a symmetrically substituted phenyl ring. The doublet resonances observed at $\delta$ 7.76 and 6.88 in the $^1$H NMR arose from two pairs of adjacent aromatic protons on a para-disubstituted phenyl ring (2″/6″ and 3″/5″). The two singlet resonances at $\delta$ 7.89 and 7.55 arose from two sets of equivalent methine carbons situated meta to one another (2‴/6‴ and 2′/6′) on separate symmetrically tetrasubstituted phenyl rings. Long-range correlations (HMBC) allowed the assignment of the phenolic ($\delta$ 159.1) and ring attachment carbon ($\delta$ 124.1) in the para-disubstituted ring. Similarly, the assignment of the phenolic, brominated, and ring-attachment carbons within the two symmetrically tetrasubstituted phenyl rings was facilitated by the observance of long-range correlations (HMBC) from the methine protons present in these portions of the structure. The presence of a dibromophenol structural fragment in the molecule was further verified by the observance of a multiple peak cluster at *m*/*z* 463−467 (isotopic pattern indicative of two bromine atoms) in the positive ion MALDI-MS spectrum, which arose from the loss of a dibromophenol species from the parent molecular ion.

The remaining fragment of cadiolide A (**1**) had to account for an elemental composition of $C_6HO_3$ and five sites of unsaturation. A carbonyl stretching band at 1721 cm$^{-1}$ in the IR spectrum and a resonance in the $^{13}$C NMR at $\delta$ 165.5 indicated the presence of an ester functionality in the molecule. A resonance at $\delta$ 185.0 and carbonyl stretching band at 1683 cm$^{-1}$ suggested the presence of a conjugated keto carbonyl group in the structure. The remaining four carbons were observed in the $^{13}$C NMR spectrum as three quaternary olefinic resonances at $\delta$ 121.3, 155.7, and 144.6 and a methine olefinic resonance at $\delta$ 118.1. These fragments were assembled together in accordance with HMQC, HMBC, and ROESY data to give the final structure of **1**. Structure **1** was supported by HMBC correlations including that from the proton at $\delta$ 7.89 (H2‴/H6‴) to the keto carbonyl carbon resonating at $\delta$ 185.0. This fixed the position of the keto group at the benzylic position of one of the 3,5-dibromo-4-hydroxyphenyl residues. A correlation from the proton at $\delta$ 7.55 (H2′/H6′) to the quaternary carbon at $\delta$ 155.7 (C6) fixed the attachment of the other 3,5-dibromo-4-hydroxyphenyl residue to C3. Correlations from the proton at $\delta$ 7.76 (H2″/H6″) to the methine carbon at $\delta$ 118.1 and from the proton at $\delta$ 6.33 to carbons at $\delta$ 133.5 and 124.1 fixed the attachment of the para-disubstituted ring to C5. Further correlations from the proton at $\delta$ 6.33 to carbons at $\delta$ 144.6 and 155.7 fixed the relative positions of the para-disubstituted phenyl ring and one of the 3,5-dibromo-4-hydroxyphenyl residues in the structure. This left the position of the ketobenzyl group, the ester functionality, and a quaternary methine at $\delta$ 121.3 unassigned. Comparison with rubrolide A[9] (**3**) isolated from the same organism allowed the ester functionality and unassigned quaternary carbon to be assigned as part of an $\alpha,\beta$-unsaturated $\gamma$-lactone moiety. In a ROESY experiment, the resonance at $\delta$ 6.33 (H5) induced a NOE in the resonance at $\delta$ 7.55 (H2′/H6′) and 7.76 (H2″/H6″), indicating that the geometry of the $\Delta^{4,5}$ olefin was in the *Z* configuration. The complete structure was therefore assigned as **1**.

Cadiolide B (**2**) was obtained as an orange amorphous solid that yielded abundant ions in the *m*/*z* 870−880 region in both the negative ion ES-MS and MALDI-MS spectra. An isotopic packet corresponding to the presence of six bromine atoms was observed (Figure 2, the top part is the measured mass spectrum, the bottom portion is

**Table 1. $^{13}C$ and $^{1}H$ NMR Data**

| | cadiolide A (**1**)[a] | | | | cadiolide B (**2**)[a] | | | |
|---|---|---|---|---|---|---|---|---|
| atom | $^{13}C$[b] | $^{1}H$ | | HMBC correlations | $^{13}C$[b] | $^{1}H$ | | HMBC correlations |
| 1 | 165.5 | | | | 165.1 | | | |
| 2 | 121.3 | | | | 122.6 | | | |
| 3 | 155.7 | | | | 155.6 | | | |
| 4 | 144.6 | | | | 146.3 | | | |
| 5 | 118.1 | 6.33 | 1H, s | 3, 4, 1″, 2″/6″ | 114.2 | 6.43 | 1H, s | 3, 4, 2″/6″ |
| 6 | 185.0 | | | | 185.1 | | | |
| 1′ | 122.7 | | | | 122.2 | | | |
| 2′/6′ | 132.9 | 7.55 | 2H, s | 3, 1′, 2′/6′, 3′/5′, 4′ | 134.0 | 7.53 | 2H, s | 3, 1′, 2′/6′, 3′/5′, 4′ |
| 3′/5′ | 111.7 | | | | 111.7 | | | |
| 4′ | 152.4 | | | | 152.5 | | | |
| 1″ | 124.1 | | | | 127.4 | | | |
| 2″/6″ | 133.5 | 7.76 | 2H, d, *J* 8.5 | 5, 2″/6″, 4″ | 134.7 | 8.14 | 2H, s | 5, 1″, 2″/6″, 3″/5″, 4″ |
| 3″/5″ | 116.2 | 6.88 | 2H, d, *J* 8.5 | 1″, 3″/5″, 4″ | 112.0 | | | |
| 4″ | 159.8 | | | | 152.2 | | | |
| 1‴ | 128.9 | | | | 129.6 | | | |
| 2‴/6‴ | 133.7 | 7.89 | 2H, s | 6, 2‴/6‴, 3‴/5‴, 4‴ | 133.7 | 7.96 | 2H, s | 6, 1‴, 2‴/6‴, 3‴/5‴, 4‴ |
| 3‴/5‴ | 111.6 | | | | 111.3 | | | |
| 4‴ | 157.4 | | | | 155.6 | | | |
| 3 × OH | | 10.26 | 3H, br s | | | 10.59 | 3H br s | |

[a] DMSO-$d_6$ as solvent and internal standard. *J* values are reported in hertz, and chemical shifts are given in $\delta$ units. All experiments were recorded at 500 MHz except the $^{13}C$ NMR spectra, which were performed at 125 MHz. [b] Carbon assignments are consistent with data obtained from DEPT spectra.

the calculated mass spectrum for the suspected compound $C_{24}H_9Br_6O_6^-$). Note the good agreement in the relative abundances of the isotopic peaks. The most abundant ion was measured to be *m*/*z* 872.5479, which corresponds within 3.8 ppm to the most abundant calculated ion for $C_{24}H_9{}^{79}Br_3{}^{81}Br_3O_6^-$ at *m*/*z* 872.5446. This is consistent with the molecular formula $C_{24}H_{10}{}^{79}Br_3{}^{81}Br_3O_6$ possessing 17 degrees of unsaturation. Fragment ions were observed at *m*/*z* 790−797 corresponding to a loss of bromine from this compound. The $^{13}C$ NMR spectrum was similar to that of **1** and contained only 18 resonances, again implying that there was some element(s) of symmetry present in a highly unsaturated aromatic structure and that the structure of **2** was quite similar to that of **1**. The formula differed simply by the replacement of two hydrogen atoms with bromine atoms. The $^{1}H$ NMR spectrum of **2** was similar to that of **1** but lacked the distinctive resonances arising from the para-disubstituted phenyl ring. Instead, the spectrum contained three signals arising from pairs of identical hydrogens ($\delta$ 8.14, 7.96, and 7.53) and a deshielded signal arising from an isolated methine hydrogen ($\delta$ 6.43). Inspection of both HMQC and HMBC NMR data allowed the assignment of the structure to proceed in a similar manner as for **1** with the exception of one phenol ring. Long-range (HMBC) correlations observed from the proton at $\delta$ 8.13 showed that both the substitution pattern and plane of symmetry in the ring were the same as the other tetrasubstituted rings in the structure. A correlation to the carbon resonating at $\delta$ 114.0 fixed the position of this ring in the structure of **2**. In a ROESY experiment, the resonance at $\delta$ 6.43 (H5) induced a NOE in the resonance at $\delta$ 7.53 (H2′/H6′) and 8.14 (H2″/H6″), indicating that the geometry of the $\Delta^{4,5}$ olefin was in the *Z* configuration. The structure was therefore assigned as **2**.

Also isolated was the known compound rubrolide A (**3**).[9] The compound was isolated predominantly in the form of the *Z*-geometrical isomer at the $\Delta^{4,5}$ double bond. Measurement of the $^{1}H$ NMR spectrum of an *E*/*Z* mixture of the two isomers in $CD_3OD$ showed approximately a 1:3 mixture. Consequent measurement of the $^{1}H$ NMR spectrum of the same sample in DMSO-$d_6$ showed a decrease in the amount of the *E* isomer. Measurement of the $^{1}H$ NMR spectrum of the same sample in $CD_3OD$ again showed that the ratio of the two isomers did not revert back to that originally observed in $CD_3OD$ but remained the same as it was in DMSO-$d_6$. The double-bond isomers were characterized using a ROESY experiment on a solution of the *E*/*Z* isomers in DMSO-$d_6$. H2 of the *Z* isomer showed an NOE enhancement with H2′/H6′, and H5 showed a correlation with both H2′/H6′ and H2″/H6″. H2 of the *E* isomer again showed an NOE enhancement with H2′/H6′, but H5 this time only showed a correlation with H2″/H6″. A correlation was also observed between H2′/H6′ and H2″/H6″, indicating that the two phenyl rings were much closer to one another in this isomer, consistent with the double bond having *E* geometry. H2′/H6′ and H2″/6″ are considerably more shielded in the *E* isomer than they are in the *Z* isomer, consistent with the two phenyl rings being in close proximity. Conversely, H5, in a shielded environment close to the single-primed ring in the *Z* isomer, has a higher chemical shift in the *E* isomer where it is pointing out into space. H2 has a similar chemical shift in both isomers (see Table 2).

**Table 2. Comparison of the Proton Chemical Shifts between the *Z* and *E* Isomers of Rubrolide A (3)**

| atom | *Z* isomer[a] | *E* isomer[a] |
|---|---|---|
| 2 | 6.61 | 6.67 |
| 5 | 6.35 | 6.97 |
| 2′/6′ | 7.78 | 7.28 |
| 2″/6″ | 8.06 | 7.15 |

[a] DMSO-$d_6$ as solvent and internal standard.

During the purification process, we observed the presence of a black compound related to cadiolide B (**2**). The $^{1}H$ NMR spectrum in $CD_3OD$ contained five singlets, three accounting for two protons each ($\delta$ 8.07, 7.85, and 7.27) and two accounting for one proton each ($\delta$ 7.01 and 6.23). Also present was a broad peak that arose from four protons ($\delta$ 6.99). The $^{13}C$ NMR spectrum also contained many poorly resolved signals. The 2D NMR data clearly showed that the compound was related to cadiolide B (**2**). Both the negative ion ES and MALDI

mass spectra contained abundant ions in the *m*/*z* 1460−1470 region with an isotopic pattern suggestive of 10 bromine atoms. The mass of the most abundant ion was measured to be 1466.2665, which implied a molecular formula of $C_{41}H_{16}Br_{10}O_{10}$ (deprotonated molecular ion has a calculated mass of 1466.2409). The measured ionic masses and isotopic abundances for the cluster matched reasonably well with the theoretical ion masses and abundances for this molecular formula. Inspection of the collision-induced dissociation mass spectra generated from the molecular ion showed fragment ions corresponding to the loss of Br, 2Br, and dibromophenol. A fragment ion corresponding to cadiolide B (**2**) was also observed. This molecular formula appears to correspond to a dimer of cadiolide B (**2**) and rubrolide A (**3**). The point of dimerization is most likely by an ether linkage between C2 of rubrolide A and one of the phenolic hydroxyl groups of cadiolide B, but it is unclear which.

The cadiolides were found to be inactive against the human colon tumor cell line HCT-116, and their biological role is not known. Even though the cadiolides do not contain nitrogen, it appears to be likely that they have an amino acid biogenetic origin along with the majority of ascidian metabolites and that they are biosynthesized from three molecules of phenylalanine.

## Experimental Section

**Animal Materials.** The ascidian *Botryllus* sp. (sample no. CMI-96-53-10) was collected at Barrang Caddi (5° 4.647′ S × 119° 19.070′ E at −3 to −15 m), Indonesia, in October 1996 and identified by CMI using field guides.

**Isolation and Purification.** The *Botryllus* sp. organism was ground and exhaustively extracted with MeOH (300 mL + 250 mL), and the combined extracts were concentrated in vacuo. The dried extract (1.27 g) was redissolved in 10% aqueous MeOH (50 mL) and extracted with hexane (3 × 100 mL). The concentration of the aqueous MeOH was adjusted to 40% by the addition of water (25 mL), and the resulting solution was extracted with $CHCl_3$ (3 × 100 mL). All three phases were concentrated in vacuo and inspected by TLC and $^1$H NMR spectroscopy. The $CHCl_3$ extract (39.2 mg) contained predominantly rubrolide A (**3**), and the aqueous MeOH fraction (1.14 g) contained cadiolides A (**1**) and B (**2**). The aqueous MeOH fraction was further purified by size-exclusion chromatography (Sephadex LH-20, 40 × 700 mm, MeOH) to afford a series of colored fractions that were inspected by TLC and $^1$H NMR spectroscopy. The fraction containing the novel compounds was purified by gradient silica flash chromatography (10 × 160 mm, 0−10% MeOH in $CHCl_3$, 0.1% TFA, 10% MeOH in $CHCl_3$) to afford rubrolide A (**3**) (7.1 mg), cadiolide B (**2**) (3.4 mg), cadiolide A (**1**) (5.6 mg), and a fourth black compound related to cadiolide B (4.5 mg).

**Cadiolide A (1)**: UV (MeOH) $\lambda_{max}$ 254 ($\epsilon$ 10 100), 406 ($\epsilon$ 16 100); IR (polyethylene substrate) $\nu_{max}$ 1721, 1683 cm$^{-1}$; $^1$H and $^{13}$C NMR data shown in Table 1; −ve ES-MS *m*/*z* 720 (5), 719 (26), 718 (30), 717 (76), 716 (45), 715 (100), 714 (33), 713 (63), 712 (20), 711 (23), 639 (2), 637 (4), 635 (4), 633 (3); −ve MALDI-MS *m*/*z* 720 (9), 719 (18), 718 (18), 717 (38), 716 (20), 715 (46), 714 (18), 713 (27), 712 (6), 711 (7), 409 (100); +ve MALDI-MS *m*/*z* 722 (6), 721 (25), 720 (30), 719 (64), 718 (38), 717 (100), 716 (26), 715 (64), 714 (6), 713 (16), 467 (10), 465 (24), 463 (12); +ve HRMALDI-MS *m*/*z* 716.7398 (M + H, calcd for $C_{24}H_{13}{}^{79}Br_2{}^{81}Br_2O_6$, 716.7401).

**Cadiolide B (2)**: UV (MeOH) $\lambda_{max}$ 270 ($\epsilon$ 16 400), 388 ($\epsilon$ 24 900); IR (polyethylene substrate) $\nu_{max}$ 1756, 1684 cm$^{-1}$; $^1$H and $^{13}$C NMR data shown in Table 1; −ve ES-MS *m*/*z* 880 (2), 879 (8), 878 (10), 877 (38), 876 (25), 875 (80), 874 (29), 873 (100), 872 (33), 871 (79), 870 (18), 869 (33), 868 (7), 867 (7), 799 (1), 798 (1), 797 (3), 796 (2), 795 (6), 794 (2), 793 (6), 792 (2), 791 (4), 790 (1), 789 (1), 788 (1); −ve MALDI-MS *m*/*z* 880 (6), 879 (16), 878 (20), 877 (54), 876 (46), 875 (99), 874 (56), 873 (100), 872 (39), 871 (65), 870 (15), 869 (26), 868 (4), 867 (5), 799 (2), 798 (3), 797 (5), 796 (9), 795 (10), 794 (14), 793 (10), 792 (14), 791 (6), 790 (6), 789 (2), 788 (2); −ve HRMALDI-MS *m*/*z* 872.5479 (M − H, calcd for $C_{24}H_9{}^{79}Br_3{}^{81}Br_3O_6$, 872.5446.

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**Supporting Information Available:** $^1$H, $^{13}$C, HMQC, HMBC, and ROESY NMR spectra and LR-MALDI-MS spectra for cadiolides A (**1**) and B (**2**) (13 pages). This material is contained in libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.

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