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Utilizing DART Mass Spectrometry to Pinpoint Halogenated Metabolites from a Marine Invertebrate-Derived Fungus

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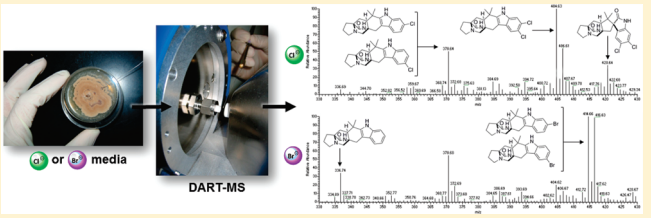
Supporting Information

ABSTRACT: Prenylated indole alkaloids are a diverse group of fungal secondary metabolites and represent an important biosynthetic class. In this study we have identified new halogenated prenyl-indole alkaloids from an invertebrate-derived *Malbranchea graminicola* strain. Using direct analysis in real time (DART) mass spectrometry, these compounds were initially detected from hyphae of the fungus grown on agar plates, without the need for any organic extraction. Subsequently, the metabolites were isolated from liquid culture in artificial seawater. The structures of two novel chlorinated metabolites, named (−)-spiromalbramide and (+)-isomalbrancheamide B, provide additional insights into the assembly of the malbrancheamide compound family. Remarkably, two new brominated analogues, (+)-malbrancheamide C and (+)-isomalbrancheamide C, were produced by enriching the growth medium with bromine salts.

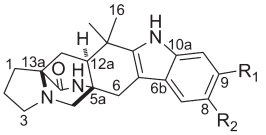


INTRODUCTION

The ever-growing group of prenylated indole alkaloid natural products is a ubiquitous biosynthetic class for fundamental investigations on many fronts. This collection contains both therapeutic and toxic compounds, and their structures, bioactivities, chemical syntheses, and biosyntheses have been the subject of research for several decades.[1] The high degree of structural diversity in this family is derived from different biosynthetic pathways to produce numerous scaffolds including dipeptides, terpenes, and aromatic polyketides. Within this group, the pattern observed for indole prenylation, such as the degree, site, and direction, adds an extra element of variety to the resulting molecules. While these compounds are commonly isolated from plants and bacteria, the majority of them originate from terrestrial and marine-derived fungi.[1]

Some noteworthy examples from fungi are those of nonribosomal peptide synthase (NRPS) biosynthetic origin including the tryprostatins,[2] fumitremorgins,[3,4] and verruculogen[5] (see Figure S1, Supporting Information). The majority of these structures are made up of tryptophan and another cyclic amino acid, condensed into a diketopiperazine (DKP) ring, and are further modified by prenyltransferase and oxidative tailoring enzymes.[6] An important subgroup of these polycyclic structures contains a bicyclo[2.2.2]diazaoctane ring system, postulated to arise from an intramolecular Diels−Alder reaction (IMDA).[7,8] Two fungal genera, *Aspergillus* sp. and *Pencillium* sp., are the source of at least 50 metabolites with this core structure, including the brevianamides,[9−11] stephacidins,[12] notoamides,[13−15] and paraherquamides[16−18] (Figure S1). The myriad of bioactivities displayed by these compounds, including antitumor, insecticidal, antibacterial, anthelmintic, and antinematodal, sustains great interest in the class.

Another subset of these structures are members of the malbrancheamide family, including the halogenated compounds (+)-malbrancheamide (**1**)[19] and (+)-malbrancheamide B (**2**),[20] and the nonhalogenated precursor, (+)-premalbrancheamide (**3**).[21] These were first isolated from a strain of *Malbranchea aurantiaca*, cultured from bat guano collected in a Mexican cave. The compounds **1** and **2** are the only prenylated indole alkaloids containing a halogenated indole ring and the bicyclo[2.2.2]diazaoctane core. Another unique characteristic is their inhibitory activity against calmodulin (CaM)-dependent phosphodiesterase (PDE1),[19,20,22] which has important implications in cancer and neurodegenerative and vascular diseases due to its effect on intracellular cAMP and cGMP concentrations.[23−26]



(+)-malbrancheamide (**1**) R_1=Cl, R_2=Cl
(+)-malbrancheamide B (**2**) R_1=Cl, R_2=H
(+)-premalbrancheamide (**3**) R_1=H, R_2=H

Since their discovery in 2006, these features have inspired several total syntheses of malbrancheamide and its analogues,[21,22,27−30] and

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two biochemical studies have emerged regarding their biosynthesis. In the first investigation researchers identified a 4-(dimethylallyl) tryptophan synthase from the genomic DNA of *Malbranchea aurantiaca*, named MaPT. It was found that this reverse prenyltransferase (PTase) enzyme was lacking the typical isoprene binding motif found in the majority of bacterial PTase enzymes, and displays high catalytic efficiency making it an attractive enzyme for synthetic biology and combinatorial biosynthesis.[31] Similarly, the reverse PTase NotF from *Aspergillus fumigatus*, involved in notoamide biosynthesis, shares these characteristics.[6] Another noteworthy study focused on the first chlorination step and timing of the reduction of the tryptophan carbonyl residue in malbrancheamide biosynthesis.[21] A key insight was gained from the transformation of the radiolabeled precursor premalbrancheamide (**3**) into malbrancheamide B (**2**) during culture of *Malbranchea aurantiaca*. It was pointed out that the protein involved in this halogenation event must bind **3** in an "exquisitely defined orientation,"[21] selectively adding a chlorine atom only to C-9, the less activated position of the indole ring. It was also postulated through the outcomes of feeding studies that reduction of the tryptophan carbonyl must take place before the Diels–Alder cyclization and formation of premalbrancheamide (**3**). This result implies that this small class of monoketopiperazine compounds is biogenetically distinct from the related diketopiperazine compounds, including the brevianamides, stephacidins, and notoamides.[21]

The unique qualities, summarized above, of this fungal genus motivated our chemical exploration of a marine invertebrate-derived strain of *Malbranchea graminicola* (collection number 086937A). Additional justification for this project was the solid tumor selectivity observed for the crude extract of 086937A in a disk diffusion assay.[32] As this work progressed, the known compounds of the malbrancheamide series (**1**, **2**, **3**) were encountered, along with four new structures in the same class. The rapid dereplication of these three known compounds and detection of new compounds was made possible by using an emerging mass spectrometry approach. We have been intrigued by the possibility of employing an open-air ion source to detect metabolites directly from a whole organism without the need for organic extraction. It seemed this would be achievable with direct analysis in real time (DART) mass spectrometry, an ion source for analysis of materials with little or no sample preparation.[33,34] DART is a versatile device that has provided ion detection of polar and nonpolar analytes directly from a variety of substances, including currency, foods, body fluids, explosives, and pharmaceuticals.[33]

It seems that this method has been overlooked by the natural products community, as the literature precedence for employing DART to profile natural metabolites is limited. Current awareness searches provided only a few examples, including detection of fatty acid methyl esters in whole cell bacteria,[35] analysis of hydrocarbon pheromones from a live fly,[36] and examination of marker compounds in herbal drugs.[37,38] Herein we show that it is also possible to detect secondary metabolites from fungal hyphae taken directly from an agar culture plate, using a DART ion source coupled to an ion trap mass spectrometer. Subsequently, milligram quantities of these metabolites were purified from the extracts of large scale liquid cultures, through selective monitoring of their molecular ions with traditional liquid chromatography–mass spectrometry (LCMS). Together these two mass spectrometry methods provided a rapid and facile route to pinpoint molecules of interest and streamline their isolation. These methods, results, and implications are discussed below.

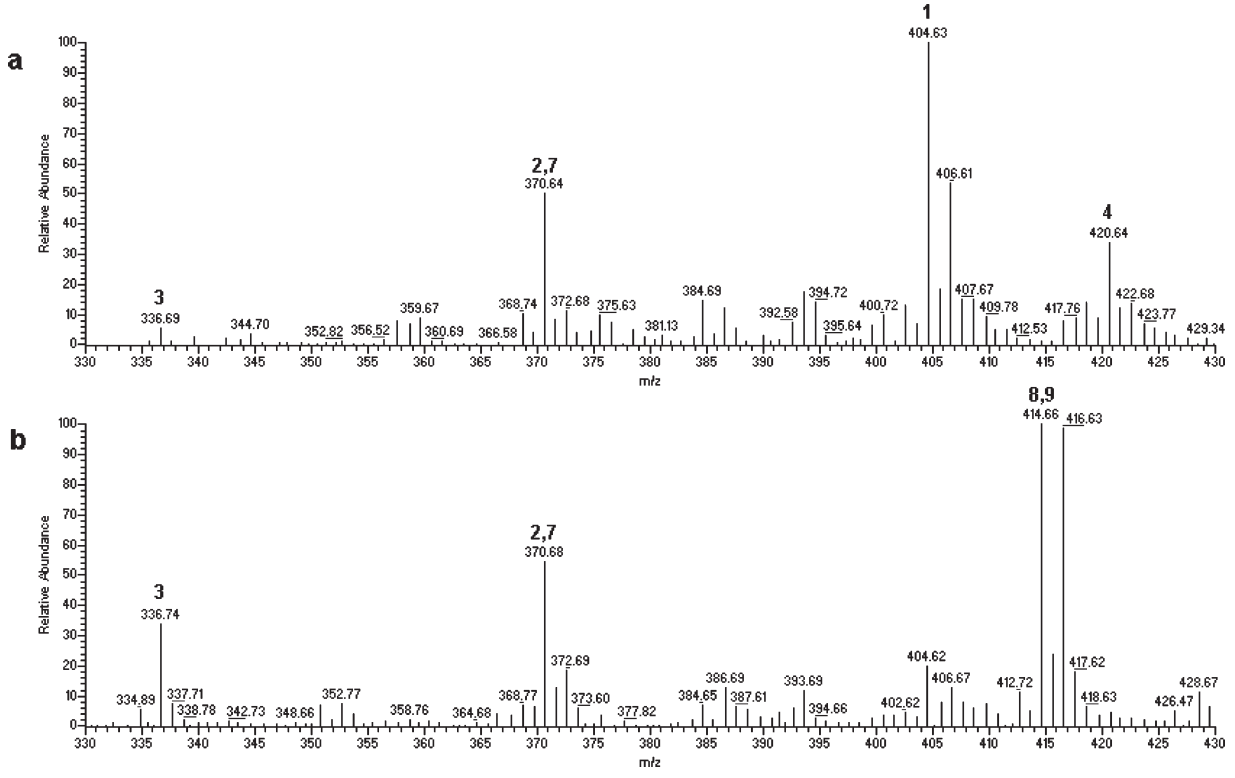


Figure 1. DART spectra of *Malbranchea graminicola* hyphae grown on two different solid agar medium types, with labeled pseudomolecular ions $[M + H]^+$ for **1**–**4** and **7**–**9**. (a) Agar medium containing chlorine salts. (b) Agar medium containing bromine salts.

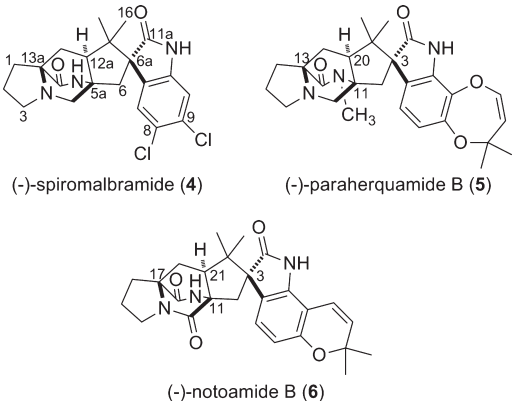
Table 1. ^{1}H and ^{13}C NMR Data (CD_3OD, 600 and 150 MHz, respectively) for (−)-Spiromalbramide (4)

	δ_C type	δ_H (J[Hz])	COSY	HMBC
1	28.3 CH_2	2.46 ddd (12.8, 9.4, 5.8)	1, 2	14
		1.45 ddd (12.2, 11.6, 5.0)		
2	22.9 CH_2	1.91 m	1, 3	1, 13a
3	54.5 CH_2	3.11 td (8.7, 2.9)	2	
		2.25 dd (9.0, 8.8)		
5	59.8 CH_2	3.71 d (11.4)	5	3, 5a, 13a
		2.65 dd (11.3, 1.5)		
5a	68.9 C			
6	39.3 CH_2	2.39 d (15.1)	6	5, 6a, 6b, 11a, 12a
		2.07 d (15.2)		
6a	63.9 C			
6b	132.3 C			
7	128.9 CH	7.52, s		6a, 8, 9, 10a
8	125.9 C			
9	133.0 C			
10	112.1 CH	7.02 s		6b, 8
10a	143.7 C			
11a	184.3 C			
12	47.1 C			
12a	55.1 CH	3.05 dd (11.0, 10.1)		13a
13	28.7 CH_2	1.86 dd (12.6, 11.2)	12a, 13	5a, 12a, 13a, 14
		1.71 dd (12.6, 10.0)		
13a	62.8 C			
14	175.9 C			
16	24.4 CH_3	0.83 s		6a, 12, 12a, 17
17	20.9 CH_3	1.12 s		6a, 12, 12a, 16

RESULTS AND DISCUSSION

The study commenced with the goal of isolating and identifying the bioactive components from culture extracts of *Malbranchea graminicola*. A pertinent first step was to culture *M. graminicola* on a solid agar medium with artificial seawater, for use in the dereplication process and for inoculation of liquid cultures. To gain an overview of the secondary metabolites produced in this organism, hyphae were removed from the agar plate using a sterile platinum inoculation loop and positioned in front of the DART source. The resulting mass spectrum from the coupled ion trap mass spectrometer is shown in Figure 1. The molecular ions for malbrancheamide (**1**) and malbrancheamide B (**2**) were easily pinpointed by their isotope patterns indicative of mono- (m/z 404.6/406.6, 1:0.6) and dichlorination (m/z 370.6/372.6, 1:0.4) patterns, shown in Figure 1a. The $[M + H]^+$ molecular ion for premalbrancheamide (**3**) was also visible (m/z 336.6) in low relative intensity. A fourth ion, observed at 420.6/422.6 (1:0.6), suggested the presence of a new dichlorinated metabolite, as no compounds with chlorine-containing molecular formulas were identified in mass-based searches (input 419.0−419.9) in relevant databases.

In order to identify the structure of this new metabolite and to obtain the malbrancheamide compounds for biological testing, a 20 L liquid culture of *M. graminicola* was prepared. Utilizing solid-phase extraction (SPE) and HPLC techniques, each of these compounds were promptly obtained from the culture extract by tracking their respective pseudomolecular ions in each purification step using ESITOFMS. Concentrating first on the



subfraction (coded H3H6) containing the 420.2/422.2 ion, the molecular formula $C_{21}H_{24}N_3O_2Cl_2$ was established and ^{1}H and ^{13}C NMR spectra were obtained. In comparison to malbrancheamide ($C_{21}H_{24}N_3OCl_2$), one additional O atom was present in the new metabolite, and while the same proton types were present (one aliphatic methine, two aromatic methines, six methylenes, and two methyls), there were clear downfield shifts of several signals in the ^{1}H NMR spectrum.

The ^{13}C chemical shifts of the new structure **4** aligned well with that of **1**, except for a carbonyl resonance at 184.3 ppm (**1**, C-11a: 145.2 ppm[19]) and a quaternary carbon at 63.9 ppm (**1**, C-6a: 104.8 ppm[19]). This data and the HMBC correlations shown in Table 1 led us to the hexacyclic structure **4**, named spiromalbramide, similar to that of (−)-paraherquamide B (**5**), with a spiro junction at C-6a. The relative configuration for **4** about the bicyclo[2.2.2]diazaoctane ring system was expected to be syn based on biosynthetic analogy to **1**,[19] and was confirmed by NOE experiments. Using the study of NOE relationships in notoamide B (**6**) as a guide,[13] the relative configuration of 5a*R**,6a*S**,12a*S**,13a*S** was established for **4** by the correlations shown in Figure 2 (also see Figure S2, Supporting Information). The absolute configuration of **4** was determined by comparing its circular dichroism spectra and Cotton effects to that reported for (+)-**5** and (−)-**5**, prepared synthetically by Cushing et al.[39] The spectra for **4** coincided with (−)-**5** (Figure 3), as was anticipated based on the negative optical rotation value obtained in MeOH ($[\alpha]^{23}_D$ −5.2), therefore supporting the assignment of 5a*R*,6a*S*,12a*S*,13a*S* configuration.

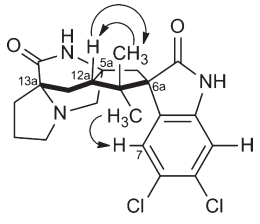


Figure 2. Significant NOE correlations, observed at 600 MHz in CD_3OD, establishing the relative configuration for (−)-spiromalbramide (**4**).

The isolation of compounds **1**−**3** was also straightforward using low resolution selective ion monitoring, and the purity of the resulting subfractions was confirmed by ^{1}H NMR. Surprisingly, two subfractions (H5H2 and H5H3) contained only the molecular ion m/z 370.2/372.2 $[M + H]^+$. While all of the ^{1}H NMR signals for fraction H5H3 matched with the literature

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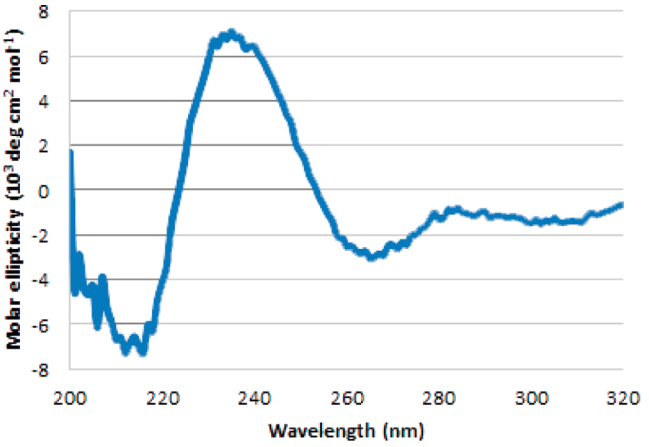
a



b

	(-)-4	(-)-5	(+)-5
λ_{max}	Δε		
200	1.7	2.3	-3.8
216	-7.2	-7.4	7.1
237	6.9	15.9	-15.3
265	-3.0	-5.6	5.0
302	-1.5	-1.8	1.2

Figure 3. (a) Circular dichroism spectrum for (−)-spiromalbramide (**4**) and (b) comparison of the Cotton effects of **4** to (±)-paraherquamide B (**5**).[39]

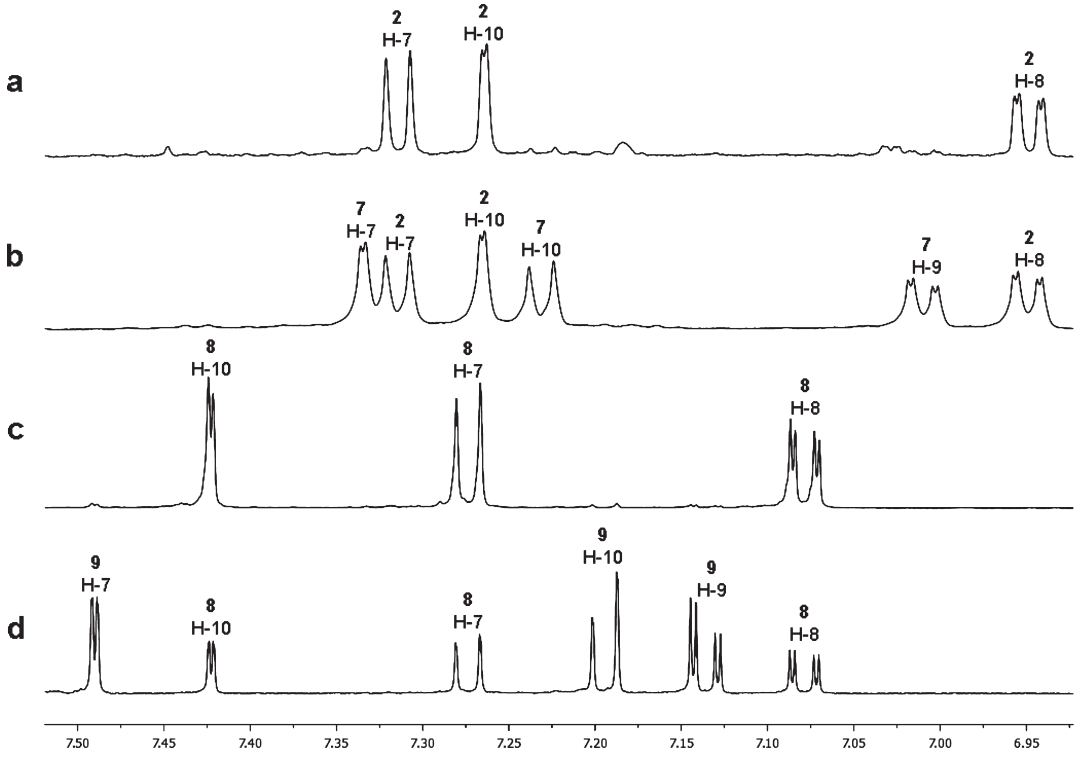
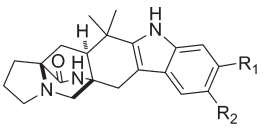


Figure 4. Overlaid ^{1}H NMR spectra (600 MHz, CD_3OD) of (a) **2** (sample code H5H3), (b) mixture of **2** and **7** (1:1, sample code H5H2), (c) **8** (sample code P3H4), and (d) mixture of **8** and **9** (0.3:1, sample code P3H3), displaying the varying indole monohalogenation patterns of the malbrancheamide family.

data for compound **2**,[20] there were three extra aromatic signals in the spectrum for H5H2 along with all of the same resonances as H5H3 (Figure 4a, 4b). The integration ratio of 0.5:1 for each of the six aromatic signals in H5H2 relative to an upfield methine signal (H-12a) proved that we had a 1:1 mixture of **2** and an isomer with a distinct aromatic substitution pattern, but otherwise identical scaffold. Through analysis of the aromatic chemical shifts, coupling constants, and HMBC correlations (Figure 5, Table 2), we proposed C-8 chlorination for the analogue **7** as opposed to C-9 in compound **2**. The ^{13}C chemical shifts for the analogue matched the reported data for a synthetic sample of isomalbrancheamide B (**7**), prepared by Miller et al., supporting this assignment.[28] Separation of these two close isomers was not possible, but we propose the same configuration for our natural sample of **7** as the rest of the malbrancheamide series, based on the positive optical rotation value for the 1:1 mixture of **2** and **7** (H5H2, $[\alpha]^{24}_D$ +12, MeOH).[40]



(+)-isomalbrancheamide B (**7**) R_1=H, R_2=Cl
(+)-malbrancheamide C (**8**) R_1=Br, R_2=H
(+)-isomalbrancheamide C (**9**) R_1=H, R_2=Br

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Table 2. ^{1}H and ^{13}C NMR Data (CD_3OD, 600 and 150 MHz, respectively) for (+)-Isomalbrancheamide B (7), (+)-Malbrancheamide C (8), and Isomalbrancheamide C (9)

	7		8		9	
	δ_C type	δ_H (J[Hz])	δ_C type	δ_H (J[Hz])	δ_C type	δ_H (J[Hz])
1	28.1 CH_2	2.54 ddd (14.3, 13.0, 6.2)	28.3 CH_2	2.55 ddd (12.6,9.0,5.6)	28.3 CH_2	2.55 ddd (12.6,9.0,5.6)
		1.47 ddd (12.6, 10.7, 7.0)		1.48 ddd (12.6,11.0,7.0)		1.48 ddd (12.6,11.0,7.0)
2	23.5 CH_2	1.89 m	23.7 CH_2	1.89 m	23.7 CH_2	1.89 m
3	55.3 CH_2	3.08 ddd (9.7, 7.4, 3.7)	55.5 CH_2	3.09 ddd (9.2,7.3,3.6))	55.5 CH_2	3.09 ddd (9.2,7.3,3.6))
		2.19 m		2.20 m		2.20 m
5	59.4 CH_2	3.47 d (10.3)	59.6 CH_2	3.47 d (10.3)	59.6 CH_2	3.47 d (10.3)
		2.29 dd (10.3, 1.7)		2.29 dd (10.3,1.7)		2.29 dd (10.3,1.7)
5a	56.0 C		57.7 C		57.7 C	
6	30.2 CH_2	2.87 d (1.1)	30.3 CH_2	2.88 d (3.5)	30.3 CH_2	2.86 d (1.6)
6a	104.5 C		105.1 C		105.1 C	
6b	129.5 C		127.3 C		130.1 C	
7	118.0 CH	7.34 d (1.9)	119.9 CH	7.27 d (8.3)	121.1 CH	7.49 dd (1.9,0.5)
8	125.4 C		122.8 CH	7.08 dd (8.3,1.7)	112.7 C	
9	122.4 CH	7.01 d (8.6, 2.1)	115.4 C		113.4 CH	7.14 dd (8.5, 1.9)
10	112.9 CH	7.23 d (8.6)	114.6 CH	7.42 d (1.7)	124.8 CH	7.20 dd (8.5,0.4)
10a	136.6 C		139.4 C		137.2 C	
11a	144.4 C		143.5 C		143.5 C	
12	35.4 C		35.6 C		35.6 C	
12a	48.6 CH	2.18 m	49.0 CH	2.17 m	49.0 CH	2.17 m
13	32.4 CH_2	2.02 dd (13.2, 11.1)	32.6 CH_2	2.02 dd (13.2,11.4)	32.6 CH_2	2.02 dd (13.2,11.4)
		1.97 dd (13.2, 5.0)		1.97 dd (13.2,5.0)		1.97 dd (13.2,5.0)
13a	66.4 C		66.3 C		66.3 C	
14	176.8 C		176.7 C		176.7 C	
16	30.7 CH_3	1.36 s	30.9 CH_3	1.34 s	30.9 CH_3	1.34 s
17	24.2 CH_3	1.45 s	24.4 CH_3	1.44 s	24.4 CH_3	1.44 s

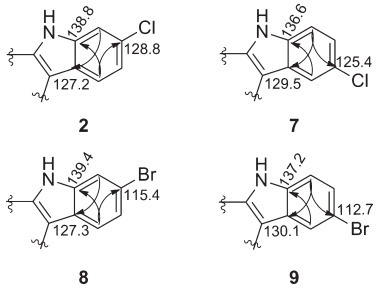

Figure 5. Significant gHMBC correlations and δ^{13}C values (C-6b, C-8 or C-9 and C-10a) for determination of indole halogenation patterns in **2**, **7**, **8**, and **9**.

The isolation of **7**, with C-8 chlorination, from our strain of *M. graminicola* was somewhat surprising, based on the previous proposal that the halogenase in *M. aurantiaca* displays complete selectivity for delivering a chlorine atom to C-9 in compound **3**.[21] Our result suggests that the halogenase in *M. graminicola* may exhibit lower specificity. This indication provided the basis for a proposal that our strain may have the ability to incorporate halogen atoms other than chlorine. To test this hypothesis, agar plates were prepared using artificial seawater enriched with bromine salts as a growth medium for *M. graminicola*. To our delight, the mass of a brominated metabolite was detected in the DART spectra from these hyphae, evident from the 1:1 isotopic ratio (m/z 414.2/416.2), as shown in Figure 1b.

A culture of *M. graminicola* was then prepared in 10 L of artificial seawater using bromine salts, with the goal of isolating the brominated analogue. Similar to the purification outcome of compound **2**, two HPLC subfractions contained the molecular mass m/z 414.2/416.2 (coded P3H3 and P3H4), and it was clear from the ^{1}H NMR spectra that both fractions contained a brominated metabolite nearly identical to compounds **1**−**3**. It was also easily established from ^{1}H NMR that P3H4 contained compound **8**, but a 0.3:1 mixture of **8** and **9** was present in P3H3, as shown in Figure 4c and 4d. The molecular formula $C_{21}H_{24}N_3OBr$ was deduced from accurate mass measurements on both fractions, and using the same NMR guidelines as mentioned above (Figure 5), the bromine placements were established in these new compounds as C-9 in malbrancheamide C (**8**, P3H4), and C-8 in isomalbrancheamide C (**9**, major isomer, P3H3). The absolute configuration shown for **8** and **9** is supported by biosynthetic analogy to compounds **1**−**3** and **7**, and by their positive optical rotation values (**8**, $[\alpha]^{24}_D$ +12.5; **9**,**8** (1:0.3), $[\alpha]^{24}_D$ +13.6, MeOH).

Our proposed biosynthesis of the malbrancheamide family of molecules is shown in Figure 6, which is based on previous biosynthetic proposals of compounds **1**−**3**,[6,21] a biochemical study of *M. aurantiaca*,[31] and the additional insights gained from the isolation of compounds **4**, **7**, **8**, and **9**. This assembly line incorporates a diverse set of putative biosynthetic enzymes, and to date only the prenyltransferase enzyme (PTase), MaPT, has been characterized

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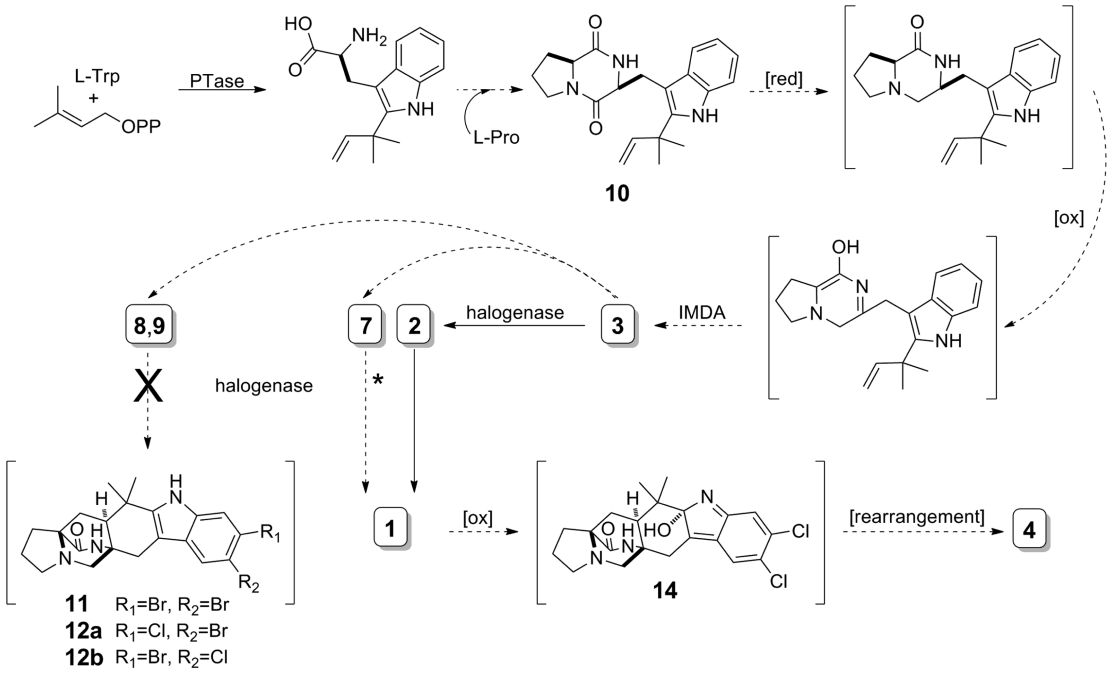
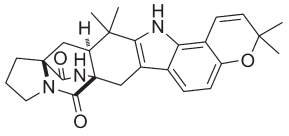


Figure 6. Proposed biosynthesis of the malbrancheamide class[6,21,31] in *Malbranchea graminicola*, including new insights from the isolation of compounds **4**, **7**, **8**, and **9**. Solid arrows represent reactions that have been confirmed with biochemical analysis or precursor incorporation experiments in *Malbranchea aurantiaca*,[21,31] and dashed arrows represent proposed biosynthetic steps. The symbol **X** indicates this step is not supported by the current study, and the symbol * indicates that this step is supported, but not proven, by this study. PTase = prenyltranferase; IMDA = intramolecular Diels–Alderase.

from *M. aurantiaca*.[31] Other postulated enzymes in the construction of malbrancheamide (**1**) include an intramolecular Diels–Alderase (IMDA), two halogenases, oxidative and reductive proteins, and a deoxybrevianamide E (**10**) synthetase.

The isolation of compounds **7**, **8**, and **9** provides evidence that the first halogenase enzyme in *M. graminicola* can incorporate more diverse substrates than previously hypothesized for *M. aurantiaca*. The integration of bromine from growth medium is documented in fungi[41,42] and other microorganisms,[43,44] and three purified tryptophan halogenases, PyrH (*Streptomyces rugosporus*), Thal (*Streptomyces albogriseolus*), and RebH (*Lechevalieria aerocolonigenes*), have been shown to incorporate both chlorine and bromine atoms.[45] However, each of these characterized proteins showed excellent regioselectivity in the monohalogenation event, unlike the results obtained here for *M. graminicola*. It seems that the second halogenase in *M. graminicola* is incapable of incorporating substrates **8** and **9**, as we did not observe a dibrominated analogue (**11**), nor a mixed chlorine and bromine congener (**12a**, **12b**). It is thus far unclear whether **7** is a substrate for the second halogenase; this matter could be resolved by feeding a radiolabeled analogue of **7** to cultures of *M. graminicola*.

The co-occurrence of compounds **1** and **4** in our culture extract suggests that a tailoring event follows the construction of **1**, thereby producing the spirooxindole functionality in **4**. Researchers have proposed that a similar transformation takes place in the notoamide-producing strain of *Aspergillus fumigatus*, based on the isolation of an analogous compound set, (+)-stephacidin A (**13**) and (−)-notoamide B (**6**).[13] The late stage conversion of **13** to **6** was postulated from a two-step sequence of oxidation followed by rearrangement.[6] Similarly we propose that the hypothetical, and undoubtedly unstable, compound **14** (Figure 6) could be formed from a stereoselective oxidation of **1**. One of two oxidative enzymes in notoamide biosynthesis in *Aspergillus fumigatus*, NotB or NotI, could be responsible for this type of oxidation.[6] Compound **14** may be the relay substrate for a complex rearrangement to **4**, and a "pinacol-type" mechanism has been hypothesized (see Supporting Information, ref 2). The elucidation of these biosynthetic enzymes would be an important advance in understanding the assembly of this intriguing class.



(+)-stephacidin (**13**)

CONCLUSIONS

There are several outcomes from this study that we consider significant. First, we have shown that DART mass spectrometry is a useful tool for detection of secondary metabolites directly from *M. graminicola* fungal hyphae, thereby circumventing the need for organic extraction of the organism and mass analysis by coupled liquid chromatography–mass spectrometry. Second, this method enabled the facile detection and rapid isolation of the new compound (−)-spiromalbramide (**4**), in conjunction with selective ion monitoring in the purification process. Additionally, by modifying the solid growth medium, the biosynthesis of novel brominated metabolites (+)-malbrancheamide C (**8** and **9**) was induced in *M. graminicola*, and these compounds were easily pinpointed by using DART-MS. This study represents one of the most useful scenarios for DART methodology, because the isotopic patterns were easily identifiable (compound **4**) or masses

of interest were known before analysis (compound **8**). Mass information obtained from DART-MS could also be important for verification of metabolite production in other microorganisms before large-scale culturing and for differentiation of seemingly identical strains. We anticipate that DART-MS will become an increasingly important dereplication tool for natural products chemists. However, utility of this method will be limited without taxonomy information that would provide inputs for database searching along with the key m/z data from DART-MS. Finally, the identification of spiromalbramide (**4**), and the monohalogenated analogues **7**, **8**, and **9**, provide chemical insight into the biosynthesis of the malbrancheamide class. We believe the genome of the marine invertebrate-derived *M. graminicola* holds important biological clues regarding the assembly of this group of prenylated indole alkaloids.

EXPERIMENTAL SECTION

General Experimental Procedures. The 1D and 2D NMR spectra were obtained using a 600 MHz spectrometer, outfitted with a cryoprobe, in CD_3OD at 600 MHz for ^1H and 150 MHz for ^{13}C. Chemical shifts are reported in ppm relative to CD_3OD (δ_H 3.31 and δ_C 49.0). High-resolution and low-resolution mass spectra were acquired with a benchtop ESI-TOF-MS. Semipreparative reverse-phase (RP) HPLC was performed using a C18 5 μm column, 10 × 250 mm, with UV peak detection and 2 mL/min flow rate. Analytical separation was achieved with a C18 5 μm column, 4.6 × 250 mm and 1 mL/min flow rate. A reverse-phase 10 μm column, 21.2 × 250 mm, was utilized for preparative RP HPLC with a 10 mL/min flow rate. The optical rotations were determined on a digital polarimeter, and the UV data were obtained on a UV−vis photodiode array spectrophotometer. The circular dichroism spectrum is reported as an average of three spectra, each measured with an integration time of 8 s per step in a 10 mm quartz cuvette. Compound purity was determined to be >95% by analytical HPLC and ^1H NMR, except when noted otherwise.

Biological Materials. *Malbranchea graminicola* (086937A) was isolated from an unidentified invertebrate collected by SCUBA in Kona, Hawaii, in December of 2008. The strain was taxonomically identified by molecular (ITS and D1/D2 regions of rDNA) and morphological methods at the University of Texas Fungus Testing Laboratory.[46] It is maintained as a cryopreserved glycerol stock at UCSC.

Culture Conditions. 086937A was grown in 20 L of artificial seawater medium (salt content per liter: 27 g of NaCl, 0.6 g of KCl, 0.3 g of $CaCl_2$, 2 g of Tris base, 7 g of $MgSO_4 \cdot 7H_2O$) containing 3.5% Czapek-Dox (086937AZa) at pH 7. A second culture of 086937A was grown in 10 L of brominated artificial seawater medium (salt content per liter: 26 g of NaBr, 0.6 g of KBr, 0.3 g of $CaBr_2$, 2 g of Tris base, 7 g of $MgSO_4 \cdot 7H_2O$) containing 3.5% Czapek-Dox at pH 7 (086937AZaBr). pH adjustments for both liquid cultures were made with 12 N HCl. Cultures were shaken at 150 rpm for 21 days at room temperature.

Extraction and Isolation. *086937AZa.* On the 21st day, ~50 g (2% volume) of prewashed XAD-16 resin was added to each flask and shaken for 12 h. The resin and mycelia were then separated from the culture broth by vacuum filtration with glass microfiber filters and a Buchner funnel. The mycelium and resin were extracted 3× with 1 L of 50% MeOH/50% DCM. The resulting crude extract (32 g) was desalted by first dissolving in 10% MeOH and adsorbing onto prewashed HP-20 resin. The resin was flushed with 1 L of 5% MeOH and then 3 L of 100% MeOH. The MeOH-soluble extract was dried, providing 2 g of desalted material. This extract was then subjected to flash chromatography using reversed-phase SPE cartridges. Eight fractions (A−H) were collected using the following solvents: A, 20% aqueous MeOH (264.3 mg); B, 40% aqueous MeOH (130.7 mg); C, 60% aqueous MeOH (128.9 mg); D, 80% aqueous MeOH (474.2 mg); E, 100% MeOH (750.2 mg); F, 70% MeOH/30% DCM (442.8 mg); G, 30% MeOH/70% DCM (54.5 mg); H, 100% DCM (7.8 mg). Fractions A, B, and C were combined based on similar LCMS profiles, and the resulting mixture was subjected to semi-preparative RP HPLC. A linear gradient (10% to 40% aqueous CH_3CN over 30 min) provided seven fractions (H1−H7). Fraction H1 was further purified by analytical HPLC using isocratic conditions (15% aqueous CH_3CN), giving **3** (H1H2, 0.7 mg). Similarly, fractions H3 and H7 were each subjected to isocratic analytical RP HPLC (20% aqueous CH_3CN) resulting in **4** (H3H6, 0.5 mg), and **1** (H7H1, 10.3 mg). Fraction H5 was also separated with the same conditions, giving **2** (H5H3, 1.0 mg) and an inseparable mixture (1:1) of **2** and **7** (H5H2, 0.9 mg).

086937AZaBr. The 10 L culture was extracted in an identical fashion to 086937AZa. The crude extract (14.6 g) was desalted, providing 2.7 g that was fractionated using the same reversed phase SPE scheme, producing eight fractions (A, 960.8 mg; B, 447.0 mg; C, 177.7 mg; D, 216.4 mg; E, 448.4 mg; F, 264.6 mg; G, 75.6 mg; H, 2.4 mg). Fractions C and D were combined based on similar LCMS profiles and then subjected to a linear gradient with preparative reversed phase HPLC (20−40% CH_3CN, 40 min). Three fractions were collected, containing semipure **3** (P1, 3.5 mg), a mixture of **2** and **7** (P2, 3.0 mg), and a semipure mixture (1.0:0.2) of **8** and **9** (P3, 4.8 mg). Fraction P3 was further purified by isocratic semipreparative RP HPLC (48% CH_3CN, 3 mL/min) giving pure **8** (P3H4, 1.9 mg) and an inseparable mixture (0.3:1) of **8** and **9** (P3H3, 0.6 mg).

*(+)-Malbrancheamide (**1**).* White solid (10.3 mg); $[\alpha]^{24}_D$ +34 (c 0.5, MeOH). Other physical properties are in accordance with published data.[19]

*(+)-Malbrancheamide B (**2**).* White solid (1.0 mg); $[\alpha]^{24}_D$ +28 (c 0.5, MeOH). Other physical properties are in accordance with published data.[20]

*(+)-Premalbrancheamide (**3**).* White solid (0.7 mg); $[\alpha]^{24}_D$ +15 (c 0.5, MeOH). Other physical properties are in accordance with published data.[21]

*(−)-Spiromalbramide (**4**).* White solid (0.5 mg); $[\alpha]^{23}_D$ −5.2 (c 0.36, MeOH). UV (MeOH) λ_{max} (log ε): 203 (3.43), 215 (3.43), 260 (2.78) nm; CD (MeOH) λ_{max} ($\Delta\varepsilon$): 200 (+1.7), 216 (−7.2), 237 (+6.9), 265 (−3.0), 302 (−1.5) nm. ^1H and ^{13}C NMR (see Table 1); HRESITOFMS $[M + H]^+$ m/z 420.12427 (calcd for $C_{21}H_{24}N_3O_2Cl_2$, 420.12401).

Inseparable mixture (1:1 ratio) of (+)-malbrancheamide B (**2**) and (+)-isomalbrancheamide B (**7**): yellow solid (0.9 mg); $[\alpha]^{24}_D$ +12 (c 0.5, MeOH). HRESITOFMS $[M + H]^+$ m/z 370.16536 (calcd for $C_{21}H_{25}N_3OCl$, 370.16807). ^1H and ^{13}C NMR for **7** (see Table 2). Other physical properties are in accordance with published data.[28]

*(+)-Malbrancheamide C (**8**).* White solid (1.9 mg); $[\alpha]^{24}_D$ +12.5 (c 0.5, MeOH). ^1H and ^{13}C NMR (see Table 2); HRESITOFMS $[M + H]^+$ m/z 414.11821 (calcd for $C_{21}H_{25}N_3OBr$, 414.11744).

Inseparable mixture (0.3:1 ratio) of (+)-malbrancheamide C (**8**) and (+)-isomalbrancheamide B (**9**): yellow solid (0.6 mg); $[\alpha]^{24}_D$ +13.6 (c 0.5, MeOH). ^1H and ^{13}C NMR (see Table 2); HRESITOFMS $[M + H]^+$ m/z 414.11948 (calcd for $C_{21}H_{25}N_3OBr$, 414.11744).

In Vitro Disk Diffusion Assay. This screen was performed as previously described[32] on the crude extract of 086937AZa. Selectivity (19.5 mm) was defined between a human prostate adenocarcinoma cell line (LNCaP[47]) versus murine macrophage cells (CFU-GM).

ASSOCIATED CONTENT

Supporting Information. NMR spectra for compounds **4**, **7**, **8**, and **9**, isolation schemes, and structures of important prenylated indole alkaloids. This material is available free of charge via the Internet at http://pubs.acs.org.

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