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Premalbrancheamide: Synthesis, Isotopic Labeling, Biosynthetic Incorporation, and Detection in Cultures of *Malbranchea aurantiaca*

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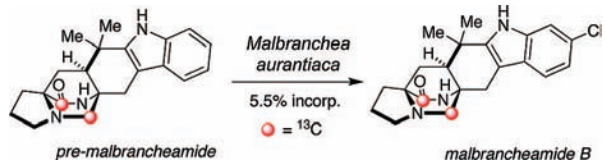
ABSTRACT



An advanced metabolite, named premalbrancheamide, involved in the biosynthesis of malbrancheamide (1) and malbrancheamide B (2) has been synthesized in double ^{13}C-labeled form and was incorporated into the indole alkaloid 2 by *Malbranchea aurantiaca*. In addition, premalbrancheamide has been detected as a natural metabolite in cultures of *M. aurantiaca*. The biosynthetic implications of these experiments are discussed.

The family of unique prenylated indole alkaloids containing a characteristic bicyclo[2.2.2]diazaoctane core has been an area of intense interest for a number of years.[1] This family of biologically active fungal metabolites include the paraherquamides,[2] brevianamides,[3] notamides,[4] stephacidins,[5] and others which are all thought to arise biogenetically from tryptophan, mevalonate-derived isoprene units, and proline or derivatives of proline.[1] Preliminary biogenetic schemes put forth by Birch and Sammes,[6] which were later supported by work from our laboratory,[1] proposed that the bicyclo[2.2.2]diazaoctane core common to all of these natural products arises in Nature via an intramolecular hetero-Diels−Alder reaction of a 5-hydroxypyrazin-2(1*H*)-one. In a continuing effort to elucidate the biogenesis of these alkaloids, we have applied such a biomimietic hetero-Diels−Alder cycloaddition strategy to the total synthesis of several of these prenylated indole alkaloids, including stephacidin A,[7] brevianamide B,[8] marcfortine C,[9] notoamide B,[7b] VM55599,[10] preparaherquamide,[10] malbrancheamide,[11] and malbrancheamide B.[11]

Within this context, the isolation of malbrancheamide (**1**) and malbrancheamide B (**2**, Figure 1) from *Malbranchea aurantiaca*, a fungus isolated on bat detritus collected in a cave in Mexico by Mata and co-workers, was significant.[12] These new substances are the first alkaloids in this class of prenylated indole alkaloids to contain a halogenated indole ring, and they are further characterized by the lack of a tertiary amide in the bicyclo[2.2.2]diazaoctane core. In addition to these notable structural features, **1** has been shown to be a calmodulin (CaM)

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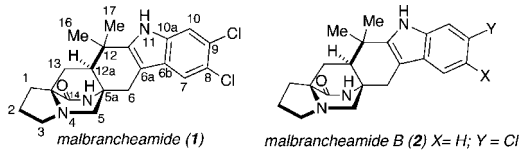


Figure 1. Malbrancheamide and malbrancheamide B.

antagonist that inhibits the activity of CaM-dependent phosphodiesterase (PDE1) in a concentration dependent manner.[12]

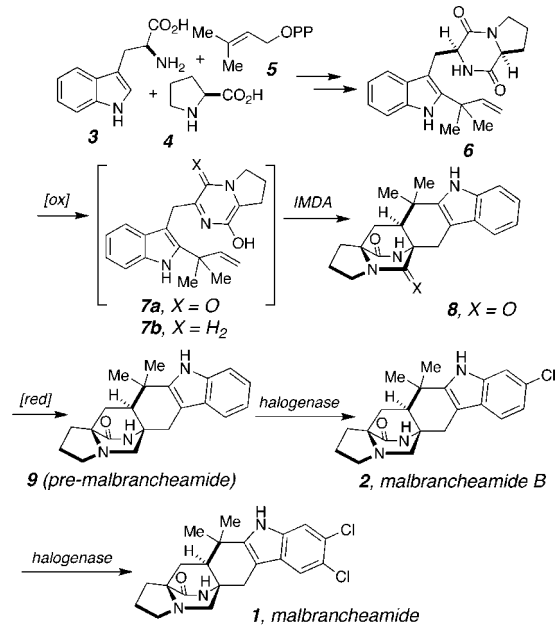
Due to their effects on intracellular cAMP and cGMP concentrations, the chemotherapeutic potential of PDE1 inhibitors includes applications in the treatment of neurodegenerative diseases, cancer, and vascular diseases.[13] Since specific PDE1 inhibitors are scarce, the study of malbrancheamide (**1**), malbrancheamide B (**2**), and their synthetic analogues is a worthy goal.

Since the malbrancheamides were the first prenylated indole alkaloids within this family to contain a halogenated indole ring, we became interested in the biosynthetic timing of the indole chlorination event. In conjunction with a program aimed at determining the effect of indole chlorination on the biological activity within this structural class, we undertook an effort to probe the biosynthetic origin of the indole C8- and C9-chlorines in malbrancheamide (**1**) and malbrancheamide B (**2**). We hypothesized that **1** arises from **9** via sequential chlorination events, with malbrancheamide B (**2**) being the result of a single, initial chlorination (Scheme 1).

As suggested above, we propose that the dioxopiperazine **6** (deoxybrevianamide E)[14] is derived biosynthetically from tryptophan (**3**), proline (**4**), and dimethylallyl diphosphate (**5**). We further propose that oxidation of **6** leads to the 5-hydroxypyrazin-2(1*H*)-one **7a**, which undergoes an intramolecular hetero-Diels−Alder (IMDA) reaction to provide **8**.[15] Reduction of the tryptophan-derived carbonyl residue of **8** would provide substrate **9**, herein named "premalbrancheamide".

Alternatively, **6** might first suffer reduction of the tryptophan-derived amide carbonyl residue leading to **7b**, whose IMDA

Scheme 1. Proposed Biosynthesis of the Malbrancheamides



reaction would directly provide **9**. To probe these questions, a number of doubly ^{13}C-labeled putative biosynthetic substrates were prepared to be employed in precursor incorporation studies. We describe herein the synthesis, isotopic labeling, and biosynthetic incorporation of the hexacyclic substance, "premalbrancheamide" (**9**), into malbrancheamide B in cultures of *M. aurantiaca*. We further demonstrate that premalbrancheamide is a natural metabolite of *M. aurantiaca* and that this substance is chlorinated at the indole C-6 position (C-9, malbrancheamide numbering), which is a less electron-rich position for (chemical) halogenation as compared to the indole C-5 position (C-8, malbrancheamide numbering).[16]

Malbrancheamide (**1**) and malbrancheamide B (**2**) have both been isolated from *M. aurantiaca*.[12] In the current study, both natural products were also found in the fungal extract by LC−MS analysis (Figure 2). The retention times of compounds **1** and **2** were 35.3 and 31.0 min, respectively, in the LC spectrum. Both compounds exhibited the expected chlorine isotope patterns and *m*/*z* values. In the MS/MS analysis, the loss of CO (C-14 atom) and then NH_3 from the molecular ion of malbrancheamide (**1**) generated fragments at *m*/*z* of 376.25 and 359.24, respectively (Figure 3). A similar fragmentation pattern was observed in the malbrancheamide B (**2**) MS/MS spectrum, further confirming the production of this natural product in *M. aurantiaca*.[12b]

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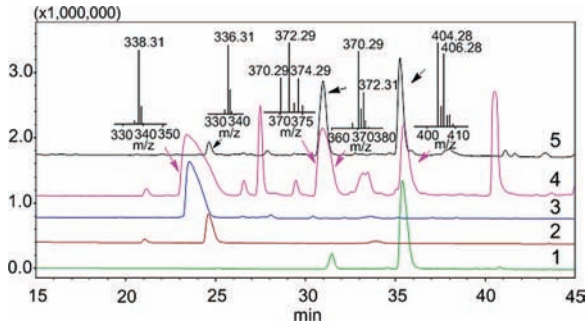


Figure 2. LC−MS analysis of extracts from *M. aurantiaca* liquid culture: lane 1, authentic malbrancheamide (**1**) and malbrancheamide B (**2**); lane 2, authentic premalbrancheamide (**9**); lane 3, doubly labeled premalbrancheamide (**17**); lane 4, *M. aurantiaca* fungal extract in feeding experiment with compound **17**; lane 5, *M. aurantiaca* fungal extract. All natural products exhibited expected *m*/*z* values in MS analysis. Premalbrancheamide (**9**) was identified in *M. aurantiaca* fungal extract by comparison to authentic compound **9**. In the feeding experiment, both ^{13}C-labeled malbrancheamide B and native compound **2** were isolated, while no ^{13}C-labeled malbrancheamide (**1**) was detected by LC−MS.

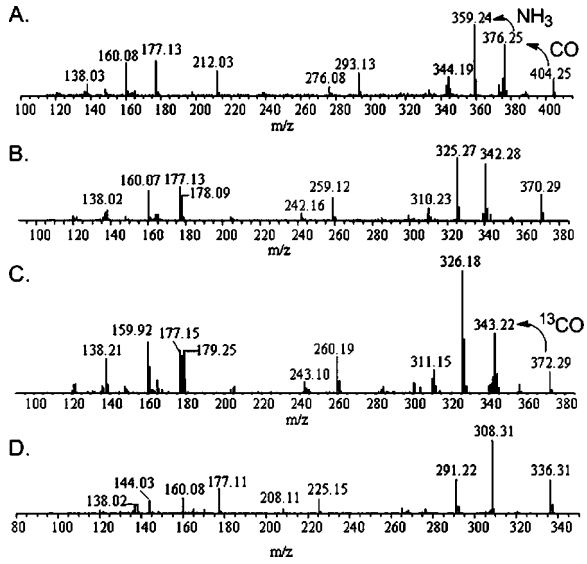
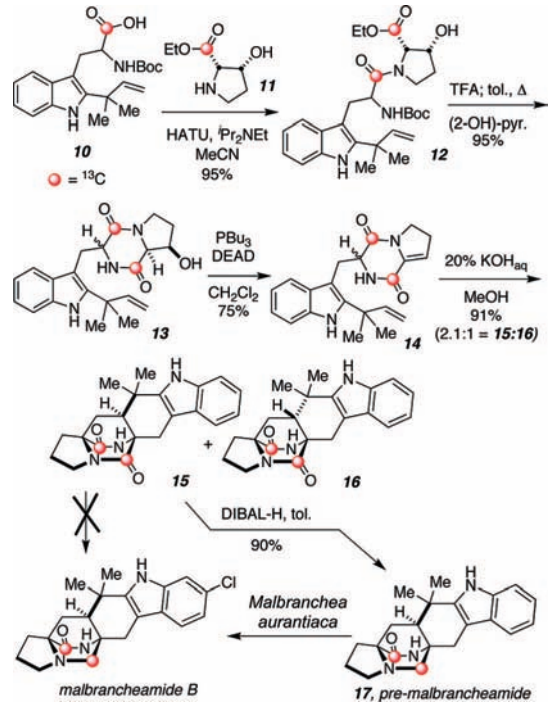


Figure 3. MS/MS spectra of malbrancheamide (**1**) (A), malbrancheamide B (**2**) (B), doubly ^{13}C-labeled malbrancheamide B (C), and premalbrancheamide (**9**) (D) from the fungal extract.

Interestingly, one compound in the fungal extract had both the same *m*/*z* value (336.31) and retention time (24.6 min) as authentic premalbrancheamide (**9**) (Figure 2). Moreover, this isolated compound had a similar MS/MS fragmentation pattern compared to malbrancheamide (**1**) and malbrancheamide B (**2**), indicative of the structural homology of these three compounds (Figure 3). Furthermore, the identical MS/MS spectra of this compound and synthetic, authentic compound (**9**) confirmed the presence of premalbrancheamide (**9**) in the fungal extract (Figure 3; Supporting Information Figure S1).

In order to investigate the role of premalbrancheamide (**9**) in malbrancheamide biosynthesis, doubly ^{13}C-labeled premalbrancheamide (**17**) was synthesized according to methods recently developed in our group in the context of the synthesis of stephacidin A[7,15d] and congeners. As shown in Scheme 2,

Scheme 2. Doubly ^{13}C-Labeled Premalbrancheamide (**17**) and Product Incorporation



amino acid coupling of the ^{13}C-labeled reverse prenylated tryptophan derivative **10** and ^{13}C-labeled *cis*-3-hydroxyproline ethyl ester (as the TFA salt) **11** in the presence of HATU provided **12** as a mixture of diastereomers. Treatment of the peptide **12** with TFA resulted in deprotection of the carbamate, and subsequent heating of the resultant primary amine with 2-hydroxypyridine in toluene gave the dioxopiperazine **13**. Dehydration of **13** under Mitsunobu conditions led to the enamide **14**, which smoothly underwent intramolecular hetero-Diels−Alder reaction when treated with aqueous KOH in MeOH affording cycloadducts **15** and **16** in a 2.1:1 ratio. Treatment of the major cycloadduct **15** with excess DIBAL-H led to selective reduction of the tertiary amide in the presence of a secondary amide to provide double ^{13}C-labeled premalbrancheamide (**17**) in excellent yield.

Compound **17** was added to cultures of *M. aurantiaca* in a precursor incorporation experiment. As a putative precursor of premalbrancheamide (**9**) (Scheme 1), compound **15** was also included in the analysis. Fungal extracts from these precursor incorporation studies were analyzed by LC−MS, and ^{13}C enrichment was revealed by MS/MS analysis.

Compound **17** was clearly incorporated intact into malbrancheamide B (**2**), whose parent ion had an *m*/*z* value of 372.29 (Figure 2). Its retention time was the same as that of the native malbrancheamide B (**2**). In the MS/MS spectrum of doubly ^{13}C-labeled malbrancheamide B (**2**), the fragment at *m*/*z* of 343.22 was produced by the loss of ^{13}CO containing a ^{13}C

atom at its C-14 position (Figure 3C). A similar fragmentation pattern was observed in the MS/MS spectrum of compound **17** (Figure S1). The *m*/*z* difference (=1) of many fragments in MS/MS spectra of labeled malbrancheamide B and natural compound **2** is due to ^{13}C atom incorporation in the fragments. From analysis of the electrospray mass spectrum, incorporation was determined to be 5.5% for the intact doubly labeled material.[17,18] Furthermore, C-5 and C-14 of the isolated malbrancheamide B had significant chemical shifts in the ^{13}C NMR spectrum, compared to compound unlabeled malbrancheamide B (see the Supporting Information, Figure S2).

Interestingly, ^{13}C-labeling of malbrancheamide itself was not detected by LC/MS−MS analysis, and only double ^{13}C-labeled malbrancheamide B (**2**) was produced in this feeding experiment (Figure 2). We tentatively believe that this is due to the kinetics of the second chlorination reaction being considerably slower than the first. Efforts are currently underway to prepare doubly ^{13}C-labeled malbrancheamide B in sufficient quantities for analogous feeding studies that we expect will show that malbrancheamide arises from a subsequent C6-chlorination of malbrancheamide B. Curiously, feeding of doubly ^{13}C-labeled dioxopiperazine **15** to *M. aurantiaca* did not label either malbrancheamide or malbrancheamide B, which again raises some important questions regarding timing of reduction of the tryptophan carbonyl residue.

In conclusion, premalbrancheamide (**9**) was isolated from *M. aurantiaca* and the identity of this substance was secured by comparison with an authentic, synthetic sample. Its role in malbrancheamide B biosynthesis was elucidated by incorporation of synthetic double ^{13}C-labeled premalbrancheamide (compound **17**) into malbrancheamide B (**2**) in *M. aurantiaca*. The regiospecific C-9 chlorination (malbrancheamide numbering) of the indole nucleus by the putative flavin-dependent halogenase[20] in the conversion of premalbrancheamide into malbrancheamide B is highly significant. It is well-known that 2,3-disubstituted indoles undergo electrophilic aromatic halogenation at the more electron-rich C-5 position (C-8, malbrancheamide numbering) in laboratory reactions.[16] We have previously prepared an authentic, synthetic sample of the corresponding C-8-monochloro regioisomer of malbrancheamide B (Figure 1, structure **2**, where X = Cl; Y = H) and did not detect a trace of this substance as a natural metabolite in *M. aurantiaca*.[11] *This clearly reveals that the halogenase must bind premalbrancheamide in an exquisitely defined orientation for delivery of the chloronium ion specifically to the less-activated C-9 position without aberrant C-8 chlorination (malbrancheamide numbering).*

The lack of incorporation of the double ^{13}C-labeled dioxopiperazine species **15** into malbrancheamide or malbrancheamide B was surprising and was further corroborated by the lack of detection of this substance as a natural metabolite of *M. aurantiaca*. *This result strongly suggests that the timing of the reduction of the tryptophan-derived amide carbonyl residue apparently precedes formation of hexacycle premalbrancheamide (**9**)*. The analogous phenomena were previously observed in our studies on the biosynthesis of paraherquamide A, where double ^{13}C-labeled substrates corresponding to **15** and **17** (except they possess a β-methyl residue in the proline ring) were prepared and only the corresponding mono-oxopiperazine species ("pre-paraherquamide") was both detected as a natural metabolite and incorporated into paraherquamide A.[17] Our findings thus indicate that the putative biosynthetic Diels−Alder construction of the bicyclo[2.2.2]diazaoctane core of this family of metabolites, which includes the paraherquamides,[2] asperparalines, marcfortines,[9] and malbrancheamides,[12] most reasonably involves a mono-oxopiperazine derived azadiene species such as **7b** (Scheme 1). The intrinsic chemistry of such species has yet to be elucidated and is the subject of current investigation in these laboratories. The facile and mild IMDA construction of hexacycle **15** from **14**, as well as more complex congeners recently reported from this laboratory,[7−9,15d] suggests that azadienes such as **7b** are expected to lead directly to premalbrancheamide (**9**). This expectation is further supported by theoretical calculations.[19] On the other hand, the structurally related families of alkaloids, which includes the brevianamides,[3] stephacidins[5] and notoamides,[4] which are constituted as dioxopiperazines, must arise by a distinct dioxopiperazine series of biosynthetic precursors.[20] This structural dichotomy is expected to manifest as a biogenetic family division whose genetic origins and biochemistry are under investigation in these laboratories and will be disclosed in due course.

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Supporting Information Available: Spectroscopic data and experimental details for the preparation of all new compounds as well as copies of ^{1}H NMR and ^{13}C NMR spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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