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Pre-malbrancheamide: Synthesis, Isotopic Labeling, Biosynthetic Incorporation, and Detection in Cultures of *Malbranchea aurantiaca*

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Abstract

total synthesis of several of these prenylated indole alkaloids, including stephacidin A,⁷ brevianamide B,⁸ marcfortine C,⁹ notoamide B,^{7b} VM55599,¹⁰ pre-paraherquamide,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) 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 analogs 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 “pre-malbrancheamide”.

Alternatively, **6** might first suffer reduction of the tryptophan-derived amide carbonyl residue leading to **7b**, whose IMDA reaction would directly provide **9**. To probe these questions, a number of doubly ¹³C-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, “pre-malbrancheamide” (**9**), into malbrancheamide B in cultures of *M. aurantiaca*. We further demonstrate that pre-malbrancheamide 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 compound (**1**) and (**2**) were 35.3 min 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 NH₃ 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}

Interestingly, one compound in the fungal extract had both the same m/z value (336.31) and the retention time (24.6 min) as authentic pre-malbrancheamide (**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 pre-malbrancheamide (**9**) in the fungal extract (Figure 3; Supporting Information Figure S1).

In order to investigate the role of pre-malbrancheamide (**9**) in malbrancheamide biosynthesis, doubly ^{13}C -labeled pre-malbrancheamide (**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, 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 pre-malbrancheamide (**17**) in excellent yield.

Compound **17** was added to cultures of *M. aurantiaca* in a precursor incorporation experiment. As a putative precursor of pre-malbrancheamide (**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 un-labeled malbrancheamide B (see 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, pre-malbrancheamide (**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 pre-malbrancheamide (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 halogenase²¹ in the conversion of pre-malbrancheamide 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-mono-chloro 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 pre-malbrancheamide 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 ¹³C-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 pre-malbrancheamide (**9**). The analogous phenomena were previously observed in our studies on the biosynthesis of paraherquamide A, where double ¹³C-labeled substrates corresponding to **15** and **17** (except possessing a β -methyl residue in the proline ring) were prepared and only the corresponding mono-oxopiperazine species ("preparaherquamide") 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 monooxopiperazine 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 pre-malbrancheamide (**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 stephacidins⁵ 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
Malbrancheamide and Malbrancheamide B

Scheme 1.
Proposed Biosynthesis of the Malbrancheamides

Figure 2.

LC-MS analysis of extracts from *M. aurantiaca* liquid culture. Lane 1, authentic malbrancheamide (**1**) and malbrancheamide B (**2**); lane 2, authentic pre-malbrancheamide (**9**); lane 3, doubly labeled pre-malbrancheamide (**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. Pre-malbrancheamide (**9**) was identified in *M. aurantiaca* fungal extract by comparing 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.

Scheme 2.
Doubly ^{13}C -labeled pre-malbrancheamide (**17**) and product incorporation.

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