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Discovery, Characterization and Analog Synthesis of Bohemamine Dimers Generated by Non-enzymatic Biosynthesis

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Abstract

Dibohemamines A–C (**5–7**), three novel dimeric bohemamine analogs dimerized through a methylene group, were isolated from a marine-derived *Streptomyces spinoverrucosus*. The structures determined by spectroscopic analysis were confirmed through the semi-synthetic derivatization of monomeric bohemamines and formaldehyde. These reactions, which could occur under mild conditions, together with the detection of formaldehyde in the culture, revealed that this dimerization is a non-enzymatic process. In addition to the unique dimerization of the dibohemamines, dibohemamines B and C were found to have nM cytotoxicity against the non-small cell lung cancer cell line A549. In view of the potent cytotoxicity of compounds **6** and **7**, a small library of bohemamine analogs was generated for biological evaluation by utilizing a series of aryl and alkyl aldehydes.

Graphical Abstract

Keywords

bohemamine; non-enzymatic biosynthesis; marine-derived *Streptomyces*; cytotoxicity; non-small cell lung cancer

There are a growing number of examples of microbial natural products that involve one or more non-enzymatic steps in the course of their biosynthesis. Examples include the jadomycins,^[1] elansolids,^[2] oxazinin A,^[3] ammosamides^[4] and discoipyrroles.^[5] For many of these families of natural products listed above, it has been possible to harness the non-enzymatic chemistry by feeding alternative precursors into the fermentation media and carrying out structure-activity relationship studies. In all of the above examples, traditional

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enzymatic biosynthesis gives rise to an electrophilic intermediate, such as an aldehyde, imine, iminium ion or *p*-quinone methide, which can then undergo a non-enzymatic reaction with a nucleophile present in the microorganisms or in the fermentation media (Figure 1). Non-enzymatic chemistry is prevalent in a variety of plant natural products, often induced by photochemical reactions. In the examples from microbes, typically a biosynthetically derived intermediate serves as an electrophile for a non-enzymatic reaction. Less obvious to identify are biosynthetically derived intermediates that behave as nucleophiles in a non-enzymatic reaction.

In our efforts to discover novel natural products from a collection of marine bacteria that have activity against non-small cell lung cancer (NSCLC) cell lines, we identified fractions from a marine-derived *Streptomyces spinoverrucosus* that had nM activity against the NSCLC cell line A549. During our examination of the active fractions, we discovered a family of known pyrrolizidine alkaloids of the bohemamine family.^[6] Although some bohemamine analogs, such as NP25301 and bohemamine (**1**), were shown to be LFA-1/ICAM-1 adhesion inhibitors,^[6c] no cytotoxicity has been reported for this family. After further examination of active fractions, we identified a series of monomeric bohemamine analogs (**2–4**)^[7] and three novel dimeric bohemamine analogs, dibohemamines A–C (**5–7**) (Figure 2), those are responsible for the biological activity. Compounds **6** and **7** showed potent activity against the NSCLC cell line A549 with IC₅₀ values of 0.140 and 0.145 μ M, respectively.

Herein, we report the isolation of the bohemamine dimers derived from fermentation and their mechanism of formation by nucleophilic addition to formaldehyde. Furthermore, we have exploited the non-enzymatic activity to generate semi-synthetic analogs for further biological evaluation.

Streptomyces spinoverrucosus strain SNB-032 was isolated from a sediment sample collected from a mudflat on a small Bahamas island and isolated on seawater based gauze 1 acidic media. From fractions that showed cytotoxicity against A549 cells, we isolated the known compounds bohemamine (**1**), bohemamine B (**2**) and 5-Cl-bohemamine C (**3**), as well as the new compound 5-Br-bohemamine C (**4**). Further examination led to the isolation of the active dibohemamines A–C (**5–7**), which had similar UV and NMR data to the bohemamines, but were more than double the molecular weight.

Dibohemamine A (**5**) was obtained as a white amorphous powder. Its molecular formula was determined as C₂₉ H₃₆ N₄ O₆ according to its HRESIMS peak at *m/z* 537.2705 [M + H]⁺. Its IR spectrum showed the presence of methylene group (2925, 1451 cm⁻¹), amide group (1640, 1565 cm⁻¹), and α,β -unsaturated ketone (1708 cm⁻¹). The UV spectrum showed three peaks at 248, 285, and 350 nm, which were nearly identical to those reported for bohemamines.^[6] The HRESIMS spectrum indicated that compound **5** should possess 29 carbons, but the ¹³C NMR only resolved 15 carbon signals (Table 1), which were classified by HSQC as four methyl carbons, one methylene carbon, four methine carbons (one oxygenated and three olefinic carbons), and six quaternary carbons (one ketone carbon). Moreover, the ¹H NMR spectrum provided the evidence for approximately half of the expected proton resonances (Table 1). The careful analysis of these data let us to suspect that

5 might be a dimeric compound. Further examination of the NMR spectra indicated that the NMR data of **5** were similar to those of the known compound bohemamine (**1**),^[6a] which was also isolated from this strain and was a major product. The bohemamine unit in compound **5** could be confirmed by the COSY correlation of H-4/H-9, and the key HMBC correlations of H-8 to C-1/C-6/C-7, H-6 to C-7, H-5 to C-6, H-4 to C-3/C-5/C-9, H-9 to C-3/C-4/C-5, 3-NH to C-3/C-1', H-2' to C-1'/C-3'/C-4'/C-5', and H-4' to C-3'/C-5' (Figure 3). The major differences between **5** and bohemamine were the loss of signals for a methine group (C-2) in bohemamine and the gain of an olefinic quaternary carbon signal (δ_C 104.3) and new methylene signals ($\delta_{C/H}$ 12.7/2.72) in **5**. The HMBC correlations of H-10 to C-1/C-2/C-3 suggested the methylene was connected with C-2 (Figure 3). Thus, it could be proposed that the methylene group served as a bridge linking C-2 and C-2'. The ESI-MS fragmentation at m/z 263 and 275 further confirmed the dimeric structure of **5** (Figures 3 and S3). The NOE correlations between H-8 and H-4 and between H-8 and H-6 (Figure 3) indicate that **5** has the same relative configuration with bohemamine.^[6d] The octant rule for cyclopentenone has been used to determine the absolute configuration of bohemamines.^[6e,8] The CD spectrum of **5** showed the same Cotton effects with **1** (Figure S1), which suggested the same absolute configuration.

Two additional dimeric structures, dibohemamine B (**6**) and C (**7**), were isolated from the active fraction. Dimers **6** and **7** are derived from reduction of the epoxides in **5** to give the corresponding diols, with **6** and **7** differing in the regiochemistry of epoxide opening. Full details on the structural determination of **6** and **7** can be found in the Supporting Information (Tables S1 and S2, Figures S2 and S3).

In addition, two monomeric, halogenated analogs (**3** and **4**, Figure 2) were isolated. 5-Cl-bohemamine C (**3**) had been previously reported from a marine-derived actinomycete.^[6d] 5-Brbohemamine C (**4**) had an MS of m/z $[M + H]^+$ of 343.0652/345.0638. The structure of **4** was determined to be a 5-bromo analog of bohemamine C based on the 1H and ^{13}C NMR data (Table S1), COSY correlations of H-9/H-4/H-5/H-6, and the key HMBC correlations of H-9 to C-4/C-5, H-6 to C-4/C-5/C-7, and H-8 to C-1/C-6/C-7 (Figure S2). We validated the structure of **4** by treatment of **1** in a $CHCl_3$ solution with 1 N HBr, giving rise to a molecule with identical NMR data.

Although it is not uncommon to find dimers of natural products,^[9] what is unusual about the structure of **5** is that the bridging methylene carbon, C-10, could not be accounted for in the monomeric unit, implying that a 1-carbon building block is involved in the dimer formation. The number of 1-carbon building blocks in nature is fairly limited, with *S*-adenosyl methionine and formaldehyde being the two most common.^[10] Based on the structure, we proposed formaldehyde was the more likely candidate in the formation of the dimeric bohemamine analogs. In order to test this hypothesis, we carried out a reaction with **1** and formaldehyde in THF. This was allowed to stir at room temperature for 48 hours. Monitoring the reaction by LC-MS, we were able to observe the addition of one monomeric unit of **1** to formaldehyde to give **8** as well as the dimer **5** (Figure 4) in a 7:1 ratio. After 72 hours, this ratio became 4:1, with little **1** remaining. **1** was stirred in media with formaldehyde for 48 hours, and the products **5** and **8** were observed (Figure S5).

The ability of the dimers to form under fermentation conditions would require that formaldehyde be produced by the bacteria. There is relatively little known about the production of formaldehyde in bacteria and no reports of formaldehyde production in actinomycetes. To determine the presence of formaldehyde in the fermentation media we used a colorimetric assay to detect/quantify formaldehyde. We determined the concentration of formaldehyde in the fermentation media of SNB-032 to be 0.22 mg/L (Figure S4).^[11] We also tested the formaldehyde in the media without any fermentation and the cultures of six additional actinomycetes in our collection, and found that the concentrations of formaldehyde in them were all less than 0.06 mg/L (Figure S4).

We propose a mechanism to the dimeric bohemamines that employs a Baylis-Hillman reaction (Scheme 1), where the electron rich C-2 of **1** acts as the nucleophile through intermediate **A** to react with the formaldehyde present in the fermentation medium to give the primary alcohol intermediate **B**. The resulting primary alcohol of intermediate **B** can be protonated under the slightly acidic conditions of the fermentation media, which then serves as a substrate for an S_N1 substitution of a second equivalent of **1**. To validate this hypothesis, we isolated **8** and treated it with 0.5% formic acid in CH₃ CN for 1 hour. The reaction proceeded to give complete conversion of dibohemamine A (**5**) (Figure 4). As the reaction only contains **8** and formic acid, this indicates the formation of **8** is reversible.

There are other examples of natural products that are produced by two monomers, where during dimerization one acts as the nucleophile and one as the electrophile. One example is the thiaspirane nuphar dimers, where Shenvi and co-workers were able to demonstrate a biomimetic approach through a sulfurizing dimerization of an enamine intermediate.^[12]

The monomeric bohemamines have previously been shown to be inhibitors of cell adhesion based on LFA-1/ICA M-1, but no cytotoxicity against cancer cell lines was found.^[6c] As part of our efforts to identify molecules that are cytotoxic to non-small cell lung cancer (NSCLC) cell lines, we screened our small collection of bohemamine monomers and dimers. We initially began by screening the collection against the four NSCLC cell lines, HCC44, HCC366, HCC1171 and A549. We found that the dimeric analogs, dibohemamines B (**6**) and C (**7**), were extremely potent against the cell line A549 with IC₅₀ values of 0.140 and 0.145 μ M, respectively. **7** also exhibited modest activity against HCC1171 with an IC₅₀ value of 1.2 μ M (Table 2).

Because of the potent cytotoxicity of **6** and **7**, we decided to take advantage of the synthetic ease of the dimerization reaction to generate a small library of bohemamine dimers. A series of aldehydes and monomeric bohemamines were utilized as starting material (Figure 5). These reactions were all carried out under slightly acidic conditions (0.5% formic acid). The aryl aldehydes, benzaldehyde, 2-pyridinecarboxaldehyde, cinnamaldehyde, and furfural, were successfully incorporated with bohemamines (**1** or **2**) to yield dimers **9–14** (Figure 5).

When we attempted to generate dimers with alkyl aldehydes containing α -methylene, such as propionaldehyde, butyraldehyde, and isopentaldehyde under similar conditions as aryl aldehydes we isolated a series of oxidized monomers **15–17** (Figure 5), that are most likely a

result of elimination of the secondary alcohol and subsequent oxidation. Repeating the reaction with propionaldehyde under an atmosphere of nitrogen led to dimer **18** (Figure 5).

We were also able to take advantage of our ability to isolate **8**, which could then be treated with bohemamine B (**2**) to give the unsymmetrical dimer **19** (Figure 5). This reaction can be used to obtain a series of unsymmetrical dimers derived from different bohemamines. Compound **8** was treated with mild hydrochloric acid and hydrobromic acid to yield dimers **20** and **21**, respectively (Figure 5).

All these derivatives (**9–21**) were tested for inhibition of the NSCLC cell lines, HCC44, HCC366, HCC1171 and A549. We found that a number of the dimeric derivatives were equally as potent to **6** and **7**. All of the active analogs contain hydroxy groups as compared to the epoxide moiety.

We have successfully isolated three bohemamine dimers dibohemamines A–C (**5–7**) from a marine-derived *Streptomyces spinoverrucosus* based on the activity screening against non-small cell lung cancer (NSCLC) cell lines. **5–7** were identified as having formaldehyde incorporated as a methylene bridge connecting two monomeric bohemamines.

The investigation on the mechanism of non-enzymatic dimerization sheds light on the expansion of the structural diversity of bohemamines, which could be exploited to generate analogs with enhanced biological activity.

Based on this theory, we utilized a series of aldehydes to produce a small library of dimeric and monomeric bohemamines (**9–21**), which could provide a basis for the further chemical and biological studies on these natural products. More broadly, an increasing number of examples of non-enzymatic steps in complex microbial natural products are coming to light.^[13] As more of these examples are reported, we can take advantage of the inherent reactivity and biosynthetic access to the reactive intermediates for generating chemical libraries around these scaffolds.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Non-enzymatic Formation of Natural Products: Electrophiles (previous work) *Non-enzymatic Formation of Natural Products: Nucleophiles (this work)*

Figure 1.
Examples of non-enzymatic biosynthesis.

Figure 2.
 Structures of isolated bohémamine analogs.

Figure 3.
Structural elucidation of **5**.

Figure 4.

A) Synthetic dimerization. B) HPLC traces at 254 nm for the formation of **5** and **8** from **1**.

Scheme 1.
Proposed mechanism of dimerization.

Figure 5.
Semi-synthetic derivatization of bohémamines.

Table 1¹H (600 MHz) and ¹³C (100 MHz) NMR data of **5** in CDCl₃

no.	δ_{C}	δ_{H} , mult. (J in Hz)
1/1''	200.0, C	
2/2''	104.3, C	
3/3''	166.5, C	
4/4''	56.5, CH	3.95, q (6.4)
5/5''	64.3, CH	3.58, d (2.9)
6/6''	56.1, CH	3.62, d (2.9)
7/7''	76.4, C	
8/8''	20.2, CH ₃	1.47, s
9/9''	14.2, CH ₃	1.32, d (6.4)
1'/1'''	164.4, C	
2'/2'''	117.6, CH	6.06, s
3'/3'''	159.3, C	
4'/4'''	20.7, CH ₃	2.26, s
5'/5'''	28.0, CH ₃	1.99, s
10	12.7, CH ₂	2.72, s
3/3''-NH		9.70, s

Table 2Cytotoxicity data against NSCLC cell lines (IC₅₀ in μM)^[a]

Compounds	A549	HCC44	HCC1171	HCC366
6	0.140	>24	3.9	>24
7	0.145	12.0	1.2	6.7
11	5.2	>24	14.3	>24
12	7.8	>24	19.7	>24
13	0.293	>24	2.5	>24
14	0.206	>24	3.4	>24

^[a]Cytotoxicity measured using CellTiterGlo. All measurements in triplicate. Compounds **1–5**, **8–10**, and **15–21** did not show cytotoxicity against these four cell lines (IC₅₀ >24 μM).