Natural Products

DOI: 10.1002/anie.201203960

A Combined Atomic Force Microscopy and Computational Approach for the Structural Elucidation of Breitfussin A and B: Highly Modified Halogenated Dipeptides from *Thuiaria breitfussi***

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The use of atomic-force microscopy (AFM) with atomic resolution shows great potential for the structural characterization of planar, proton-poor compounds, as these compounds are prone to structural corrections.[1,2] Currently, AFM has limited ability to identify element type and consequently functional groups. Additional computational techniques, such as computer-aided structure elucidation (CASE) and the calculation of ^{13}C NMR shifts using electronic structure calculations (DFT) may assist in this respect. Herein we show the combined use of spectroscopic methods, AFM, CASE, and DFT to solve the structures of breitfussins A and B, which could not be solved using either method alone.

The subject of this study was the Arctic hydrozoan *Thuiaria breitfussi* (Family *Sertulariidae*). The few publications on the chemistry of this family show the presence of sterols,[3] polyhalogenated monoterpenes,[4] and anthracenone derivatives.[5] Arctic marine environments support highly diverse and dense populations of marine invertebrates.[6,7] A diverse range of natural products has been found in cold-adapted marine invertebrates and microorganisms, but there appear to be no clear cold-water structural types.[8] Recent work on Arctic invertebrates has yielded novel structures[9] and analogues of known compounds.[10–12] The sample of *Thuiaria breitfussi* was collected from Bjørnøya (Bear island) in 2007 and extracted with MeOH/CH_2Cl_2. A series of liquid–liquid extractions followed by mass-guided HPLC purification yielded 6.2 mg of purified breitfussin A (**1**) and 4.0 mg breitfussin B (**2**) (Scheme 1) as the major compounds.

High-resolution mass spectrometry gave the molecular formula $C_{16}H_{11}N_3O_2BrI$ for (**1**) and $C_{16}H_{11}N_3O_2Br_2$ for (**2**), both with 12 double-bond equivalents. Fragmentation analysis of **1** revealed fragments corresponding to the loss of I (MS^2), CH_3O (MS^3), Br (MS^3), and CH_3 (MS^3). The ratio of heavy atoms to protons (ca. 2:1) indicated that structure determination by spectroscopic methods would be challenging.[13] ^{1}H NMR in [D_6]DMSO displayed two NH protons between δ_H 11.5 and 12.5, six aromatic ^{1}H singlets between δ_H 6.0 and 8.0, and a MeO group between δ_H 3.7 and 4.0 for both **1** and **2** (Supporting Information, Figures S3 and S8). The ^{13}C NMR spectrum showed one methyl carbon, six methines, and nine quaternary carbon atoms (Supporting Information, Figures S7 and S12, Table S2).

In [D_4]MeOH, coupling constants were consistent with a 2-substituted pyrrole moiety, which in the case of **2** was also substituted in the 5 position. Furthermore, both molecules

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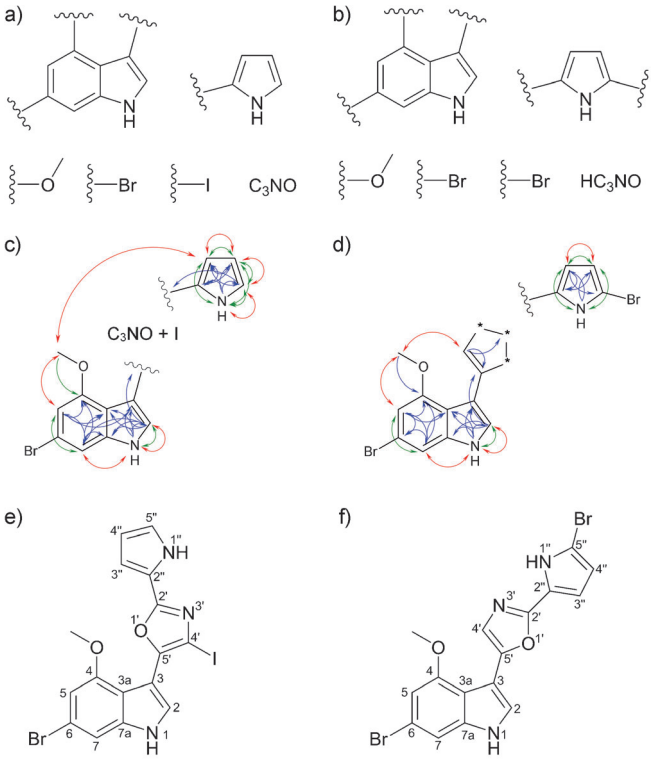
[**] MabCent is a Centre at University of Tromsø and supported by the Research Council of Norway, Grant no 174885/130. Marbank is acknowledged for collection and taxonomy of *Thuiaria breitfussi*. Financial support from EU projects ARTIST (Contract No. 243421), HERODOT (Contract No. 214954), and the ERC advanced grant CEMAS, as well as Int.PhD.P.P. of the Foundation for Polish Science co-financed from E.R.D.F. "Grants for Innovation" and Research Council of Norway by grant no. 179568/V30 and 177558/V00, are gratefully acknowledged.

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201203960.



Scheme 1. Substructures initially derived by analysis of the spectroscopic data for a) **1** and b) **2**. MS fragmentation and NMR correlations (J_{HH}, green; J_{HC}, blue; *ROE*, red) was used to connect all substructures except a C_3NO grouping plus (correlations are explicitly listed in the Supporting Information, Table S2) c) I for **1** and d) H for **2** (* = C, N, or O). The final suggested structures of e) breitfussin A (**1**) and f) breitfussin B (**2**).

had two aromatic protons in a *meta* relationship (Supporting Information, Figures S5, S6, S10, S11). Along with the pyrrole, two additional structural elements could be derived (Scheme 1 a,b): a probable 3,4,6-substituted indole and a MeO group, which could be connected to the 4 position of the indole on the basis of an NOE correlation with one of the *meta* coupled singlets (H5) but not to the other (H7). After including the halogen atoms, there were two double bond equivalents to be incorporated in the remaining C_3NO fragment. A MS^3 fragment C_9H_8BrNO (223.9707 m/z) corresponds to a brominated 4-methoxyindole. A second MS^3 fragment $C_7H_6N_2O$ (135.0551 m/z) corresponds to the remaining silent C_3NO atoms plus the pyrrole (C_4H_4N) observed by NMR spectroscopy (Supporting Information, Figure S1). A $^3J_{CH}$ coupling from the indole H2 to one of the remaining non-assigned atoms C5′ connects this grouping to the C3 of the indole and thus the Br must be attached at C6, giving the substructures presented in Scheme 1 c,d. The remaining grouping could not be unambiguously elucidated from spectroscopic data alone.

The presence of H4′ in the remaining grouping in **2** added some further insight into this moiety. The long-range carbon couplings to C5′ (weak) and C2′ (strong) indicated that the former is a $^2J_{CH}$ and the latter a $^3J_{CH}$ in a planar ring structure (Supporting Information, Figure S9). A strong ROE cross-peak between H4' and the MeO protons fixes the position of H4′. The above observations together with the characteristic chemical shift for H4′ suggested an oxazole substructure, where the exact substitution pattern with respect to the nitrogen and oxygen atoms could not be directly assessed.

Additional information to support a structural proposal was obtained using AFM with CO functionalized tips[14] on individual molecules of **1** adsorbed on Cu(111) (Figure 1).

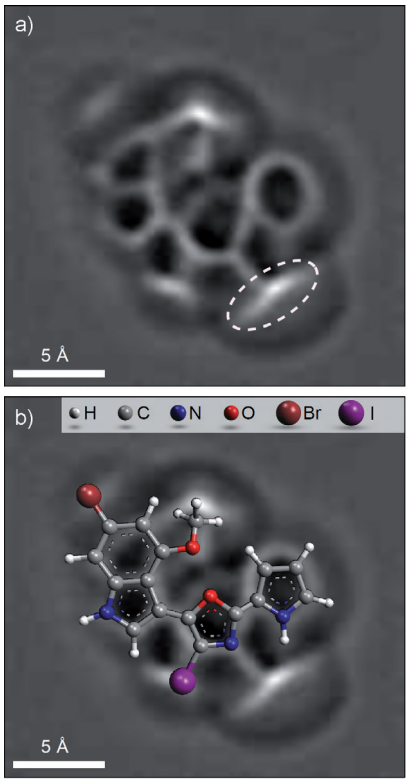


Figure 1. a) Low-pass and Laplace filtered constant height frequency shift maps of **1** on Cu(111) using AFM with CO tip functionalization. The white encircled region marks a non-intrinsic molecule feature (see the Supporting Information). b) AFM image of **1** overlaid with the proposed structure.

Atomically resolved AFM measurements revealed a bicyclic system that includes a 5-membered ring and two additional connected rings where the symmetry could not be resolved unambiguously owing to the heteroatoms. Thus, the indole fragment could be readily confirmed. Furthermore, the connection points of the rings at 3,5′ and 2′,2″ could be determined. Br and I are imaged as elongated single spots, which could be the result of their anisotropic electron distribution. The halogens are connected at 6 and 4′ or vice versa to the ring system. Furthermore, we could allocate the major spot in the upper part of Figure 1 a as the bulky MeO moiety. Consequently, the structure illustrated in Figure 1 b is consistent with the MS, NMR, and AFM experimental data. Note that I is imaged with increased contrast compared to Br, which can be explained by the additional filled shell and the corresponding larger atomic radius of I compared to Br. The prominent spot encircled in Figure 1 a is not attributed to an intrinsic molecular feature, as it disappeared after the

molecule was moved with the tip and it did not appear consistently on every individual molecule that was scanned (Supporting Information, Figure S19).

ACD/Structure Elucidator was utilized by enumerating all possible structures given chemical shifts and coupling restraints from NMR (Supporting Information, Table S3). The resulting structures were ranked on the basis of chemical shift prediction methods.[15] Two iterations of CASE analysis were performed (see the Supporting Information). The first run included several NMR-derived constraints but did not yield structures that agreed with the AFM images. A second run was performed with less manually introduced constraints, and the resulting highest-ranked structure was identical to **1** (Supporting Information, Figures S13–S16). This confirmed the proposed oxazole moiety and the placement of I at C4′ is consistent with the observed ^{13}C upfield diamagnetic shift.[16]

The only remaining possible ambiguity is the practically unlikely but theoretically possible switch of the oxazole nitrogen and oxygen positions, which cannot be directly assessed by any of the above methods. Although the CASE analysis ranks the final suggested structures higher than any other of the six possible configurations of this ring for **1** and four for **2** (Supporting Information, Scheme S1), there is an intrinsic risk of bias towards known structures owing to database coverage. To rule this out, relativistic four-component DFT chemical shift calculations[17,18] were performed on all the theoretically possible reshuffled configurations of the oxazole ring. The calculated chemical shifts all show the least average error for the proposed structures compared to any other configuration for both ^{13}C and ^{1}H shifts and for both **1** and **2** (Supporting Information, Tables S4–S7). Thus, both DFT calculations and the database approach are in agreement with respect to the silent atoms in the oxazole.

Structurally, the breitfussins comprise a rare molecular framework, with the combination of an indole, oxazole, and a pyrrole. The diazonamides, isolated from an ascidian of the genus *Diazona,* contain an indole–oxazole moiety, halogenated on the 2 position of the indole and the 4 position of the oxazole.[19] Simpler compounds containing the indole–oxazole grouping have been isolated from red algae[20,21] and bacteria.[22,23] All of the structures contained few protons, and thus the structure clarification was achieved using X-ray crystallography. The structure of almazole D[20] was proposed using NMR data and an X-ray structure of an analogue, but was later corrected by synthesis.[24] The only reported instance of the oxazole–pyrrole unit is the polychlorinated phorbazoles isolated from the sponge *Phorbas* sp., which was solved using X-ray crystallography.[25] The halogenation in breitfussin A is unusual, with an iodinated oxazole not having been reported previously. The breitfussins and phorbazoles likely share a common biogenesis, from the dipeptides Pro–Trp and Pro–Tyr, respectively. The peptide bond is converted into the oxazole, followed by oxidation of the Pro to pyrrole and final tailoring (Supporting Information, Figure S17).

In conclusion, this study presents the first compounds from the hydrozoan *Thuiria breitfussi*. The breitfussins are unusual structures containing a combination of indole–oxazole–pyrrole units. Given the limited quantity isolated, X-ray crystallography was not possible, and structure determination using spectroscopic data was unable to propose a single unequivocal structure consistent with all the data. The structure was therefore supported by a combination of AFM, CASE, and DFT calculations, none of which were able to propose a unique solution individually. Remarkably, AFM could be used to determine all the connection positions of the cyclic systems as well as those of the substituent groups (MeO, Br, and I); this information is difficult to obtain with other techniques. Moreover, different AFM contrast above halogen atoms and the MeO group indicate chemical sensitivity within individual molecules. Using such powerful methods on limited quantities of bioactive natural products with complex structures will become more common as scientists begin to access unusual taxa from extreme locations with unique chemistry.

Experimental Section

Breitfussin A (**1**; 5-(6-bromo-4-methoxy-1H-indol-3-yl)-4-iodo-2-(1H-pyrrol-2-yl)oxazole): ^{1}H NMR (600 MHz, [D$_6$]DMSO) $\delta =$ 11.95 m (H1″); 11.78 d, $J = 2.8$ Hz (H1); 7.65 d, $J = 2.7$ Hz (H2); 7.28 d, $J = 1.5$ Hz (H7); 6.96 m (H5″); 6.73 d, $J = 1.5$ Hz (H5); 6.68 m (H3″); 6.20 m (H4″), 3.77 s ppm ($H4_{MeO}$). ^{13}C NMR (151 MHz, [D$_6$]DMSO) $\delta = 157.37$ (C2′), 154.09 (C4), 146.86 (C5′), 138.33 (C7a), 127.74 (C2), 122.75 (C5″), 119.66 (C2″), 115.69 (C6), 115.48 (C3a), 110.41 (C3″), 110.01 (C4″), 108.39 (C7), 104.75 (C5), 101.19 (C3), 84.42 (C4′), 56.17 ppm ($C4_{MeO}$). HRESIMS *m*/*z* 483.9163 $[M+H]^+$ (calcd for $C_{16}H_{12}N_3O_2BrI$ 483.9158).

Breitfussin B (**2**; 2-(5-bromo-1H-pyrrol-2-yl)-5-(6-bromo-4-methoxy-1H-indol-3-yl)oxazole): ^{1}H NMR (600 MHz, [D$_6$]DMSO) $\delta = 12.54$ t, $J = 2.5$ Hz (H1″); 11.73 d, $J = 2.7$ Hz (H1); 7.77 d, $J = 2.6$ Hz (H2); 7.35 s (H4′); 7.25 d, $J = 1.5$ Hz (H7); 6.73 d, $J = 1.5$ Hz (H5); 6.71 dd, $J = 3.7$, 2.6 Hz (H3″); 6.26 dd, $J = 3.7$, 2.3 Hz (H4″); 3.95 s ppm ($H4_{MeO}$). ^{13}C NMR (151 MHz, [D$_6$]DMSO) $\delta = 154.09$ (C4), 153.42 (C2′), 146.46 (C5′), 138.94 (C7a), 124.07 (C2), 123.16 (C4′), 122.27 (C2″), 115.79 (C6), 113.14 (C3a), 112.12 (C4″), 111.20 (C3″), 108.47 (C7), 104.47 (C5), 104.39 (C3), 102.40 (C5″), 56.03 ppm ($C4_{MeO}$). HRESIMS *m*/*z* 435.9301 $[M+H]^+$ (calcd for $C_{16}H_{12}N_3O_2Br_2$ 435.9296)

Received: May 22, 2012
Revised: June 28, 2012
Published online: October 26, 2012

Keywords: atomic force microscopy · breitfussin · density functional theory · structure elucidation · *Thuiaria breitfussi*

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