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# Mass-tagged aminated probes for rapid discovery of azaphilic natural products in fungal crude extracts

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Reactivity-based screening (RBS) was used to screen fungal crude extracts for the presence of new azaphilic natural products. A probe composed of a nucleophilic primary amine and an isotopic mass tag was designed for its reactivity towards azaphilic compounds. Addition of the probe to crude extracts of fungal complexes, together with analytical tools for dereplication such as haloseeker and molecular networking, allowed easy detection of azaphilic compounds. The exploitation of this RBS protocol led to the isolation and structural characterization of new azaphilones.

The Natural Products (NPs) encompass structures ranging from simple to highly complex, showcasing diverse biological properties. Despite being consistently successful as sources of drug leads, their study and development in the pharmaceutical industry have markedly declined since the 1990s.<sup>1</sup> This is mainly due to the costly- and time-consuming traditional bioactivity-guided isolation steps that lead to original bioactive compounds from complex crude extracts. To address this, processes were devised to rapidly identify and exclude known compounds in order to focus on novel substances.<sup>2</sup> However, such dereplication is inherently limited by the size and the quality of the available databases and it still remains a work-intensive approach. In this context, Reactivity-based screening (RBS) – the chemical labeling of a specific functional group in a natural product – is an attractive method to overcome the challenges related to poor detectability, low availability and chemical instability. This approach has recently emerged and probes targeting hydroxyls<sup>3</sup>, electrophilic natural products,<sup>4,5,6</sup> alkynes<sup>7,8</sup> or isonitrile<sup>9</sup> have been developed. A noteworthy class within electrophilic natural products is represented by the azaphilone family, boasting a reactive skeleton towards primary amines, forming covalent adducts. With over 600 described members<sup>10</sup>, including compounds like epicocconone (linear lactone), chaetoviridin A (angular lactone) or mitorubrinic acid (ester), the azaphilone family offers a rich diversity,

presenting opportunities for the development of efficient probe enabling the capture and identification of new members (Figure 1).

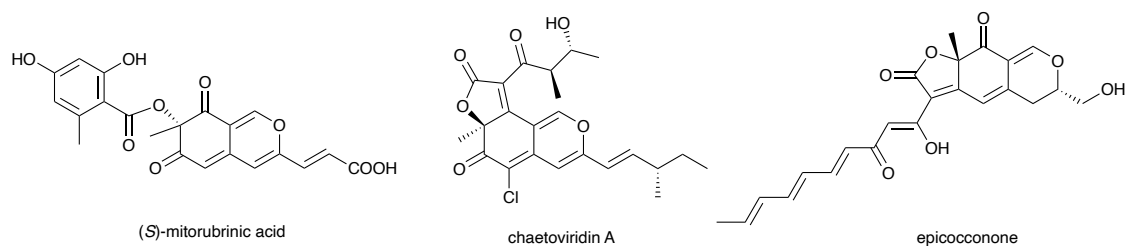


Figure 1. Azaphilone compounds

We therefore thought that the use of a probe consisting of a nucleophilic primary amine and a mass tag would allow easy detection of the probe-azaphilone or probe-azaphilic NP adduct. We thus synthesized probe 1 with a permanent charge (phosphonium ion) as MS tag to increase the MS signal-to-noise ratio and with a bromine atom for isotopic labelling. Thanks to the available azaphilone database<sup>10</sup> and MS/MS fragmentation analysis by molecular networking, we could easily determine if new azaphilones were captured (Figure 2).

Here, we applied the RBS strategy to the detection and identification of azaphilic compounds in the fungal extracts of *Talaromyces flavus* var. *flavus* (CBS 354.72B) and *Bartalinia robillardoides*, two fungi known to be producers of azaphilones.<sup>10,11,12</sup> Probe 1 was thus added to a *Talaromyces flavus* crude extract and adducts were monitored by LC-MS for the presence of the probe-tag bromine isotope pattern. HaloSeeker 1.0 was used to automatically search for the bromine signatures in full-scan high-resolution mass spectrometry (HRMS) fingerprints.<sup>13</sup> The HaloMap obtained from HaloSeeker allowed probe-azaphilone adducts to be identified. Dereplication by searching in the azaphilone database<sup>10</sup> allowed rapid identification of the presence of mitorubrinol, mitorubrinic acid, 6''-hydroxy-mitorubrinic acid together with a putative unknown tagged azaphilone at  $m/z$ : 1268.3577, corresponding to a native azaphilone at  $m/z$ : 811.1865. Another compound at  $m/z$ : 825.1663 (tagged azaphilone at  $m/z$ : 1308.3542) was also depicted and could correspond to the putatively assigned diazaphilonic acid 2 according to its chemical formula (C<sub>42</sub>H<sub>32</sub>O<sub>18</sub>) (Figure 3).

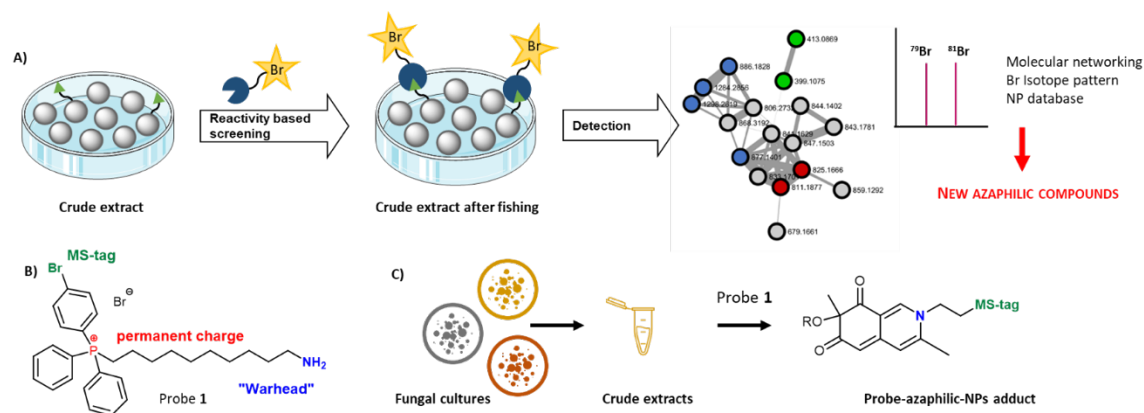


Figure 2. Azaphilones and capture strategy. A) RBS screening and dereplication step leading to the isolation of new azaphilic compounds. B) Charged aminated probe bearing mass-tag. C) RBS for azaphilic compounds with probe 1



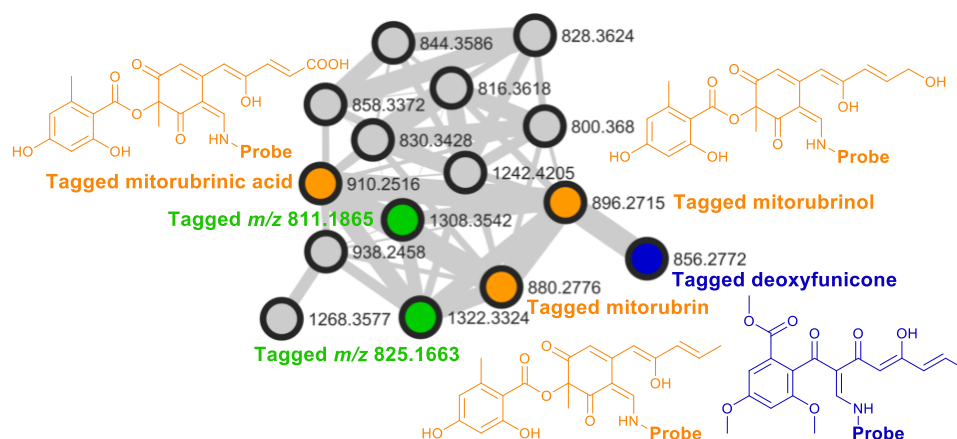


Figure 5. Cluster of tagged azaphilic compounds with probe 1 from the crude extract of *Talaromyces flavus* obtained by FBMN. In orange tagged known azaphilones, in green tagged unknown azaphilones, in blue tagged known azaphilic compound non related to azaphilone.

To confirm the identification of compound (2) at  $m/z$ : 825.1663 as diazaphilonic acid and to characterize the compound (3) at  $m/z$ : 811.1865, both were specifically sought by MS-guided isolation along with the known azaphilones and successively fully elucidated by NMR and ECD. Compound (2) was obtained as a red amorphous powder and assigned a molecular formula of  $C_{42}H_{32}O_{18}$  based on the HRESIMS peak at  $m/z$   $[M+H]^+$ : 825.1663 (825.1668 calcd for  $C_{42}H_{33}O_{18}$ ). The IR spectrum at 3360 and 1720  $cm^{-1}$  featured absorption bands typical of alcohol and carboxylic acid, respectively.  $^1H$  and  $^{13}C$  NMR data confirmed the identification of (2) as the diazaphilonic acid putatively described by Tabata12b (Table S2). The relative configuration of the cyclohexene ring was determined from the  $^1H$  J values. The absence of a coupling constant between H12a and H13a and between H13a and H13 suggested that all three protons were in a pseudo-equatorial configuration. The C8 and C8a stereogenic centres were assigned in R configurations due to the negative specific rotation of the monomer mitorubrinic acid isolated from the crude extract of *T. flavus*.<sup>16,17</sup> We then used TDDFT to calculate the ECD of the two possible stereoisomers, (8R, 13R, 8aR, 12aR, 13aR)-2 and (8R, 13S, 8aR, 12aS, 13aS)-2, for comparison with experimental CD. The calculated ECD data indicated that the positive cotton effect at 300 nm was associated with the R configuration of the C8/C8a chiral centres, further supporting our biosynthetic assignment from the monomer. The calculated spectra of the two remaining stereoisomers displayed significant differences allowing a final assignment of (8R, 13R, 8aR, 12aR, 13aR)-2 from the close match between the experimental and calculated spectra (Figure S45). Compound (3) was obtained as a red amorphous powder and was assigned the molecular formula of  $C_{42}H_{34}O_{17}$  on the basis of the HRESIMS peak at  $m/z$   $[M+H]^+$ : 811.1865 (calcd 811.1874 for  $C_{42}H_{35}O_{17}$ ). The IR spectrum of (3) featured typical absorption bands for alcohol (3360  $cm^{-1}$ ), ester (1760  $cm^{-1}$ ) and carboxylic acid (1720  $cm^{-1}$ ). Analysis of the 1D and 2D NMR spectra revealed the presence of two monomeric mitorubrinic acid and mitorubrinol moieties for (3). The  $^1H$ - $^1H$  COSY spectrum of (3) highlighted the presence of two spin systems including H12, H13, H14 and H12a, H13a, H13 and H12 (Figure S19). In addition, key HMBC correlations between H12 and C3, between H12a and C4, C5, C3a along with the chemical shift of C3a at  $\delta_C$  103.5 ppm and of C14 at  $\delta_C$  70.2 ppm suggested that the two monomers were linked by a spiro-ketal carbon at C3a. Based on the chemical formula and the HMBC correlation between H13a and C14a, the carboxylic acid was linked to C13a.

The relative configurations were deduced from the values of the proton coupling constants (Table S2). Indeed, the very low 4JCH coupling constant between of H12a and H13 ( $J = 1.4$  Hz) suggested both protons were in an equatorial configuration. The larger coupling values for H13a (dd,  $J = 3.2, 2.3$  Hz) indicated, unlike 2, that this proton was now in an axial configuration. The NOESY correlation between H13a and H14 $\beta$  at  $\delta$ H 4.01 confirmed H13a in an axial configuration in the tetrahydropyran ring and the same configuration around the cyclohexene ring as 2. The NOESY correlations from  $\delta$ H 3.04 (H4a) to  $\delta$ H 5.83 (H6) and from  $\delta$ H 7.99 to  $\delta$ H 4.01 then allowed the relative configuration of the spiroketal to be assigned (3aR\*, 13R\*, 12aR\*, 13aS\*). We couldn't assign the relative configuration between the C8/C8a and the C3a/C12a/C13a/C13 chiral centers by NMR so we used experimental CD and calculated ECD. There was again a clear match between the experimental spectra and calculated spectra of (8R, 13R, 3aR, 8aR, 12aR, 13aS)-3 confirming the same configuration as 2. Compound (3) was thus assigned as shown in Figure 6 and named pavesiflonic acid; it might be results of a Diels-Alder reaction between mitorubrinol and mitorubric acid followed by isomerization and ketal formation (Figure 6). Carrying the Diels-Alder reaction between mitorubric acid and mitorubrinol using Lewis acids or high pressure (12 or 16 kbar), resulted only in no reaction or degradation. We therefore speculate that this process may be enzyme-assisted.

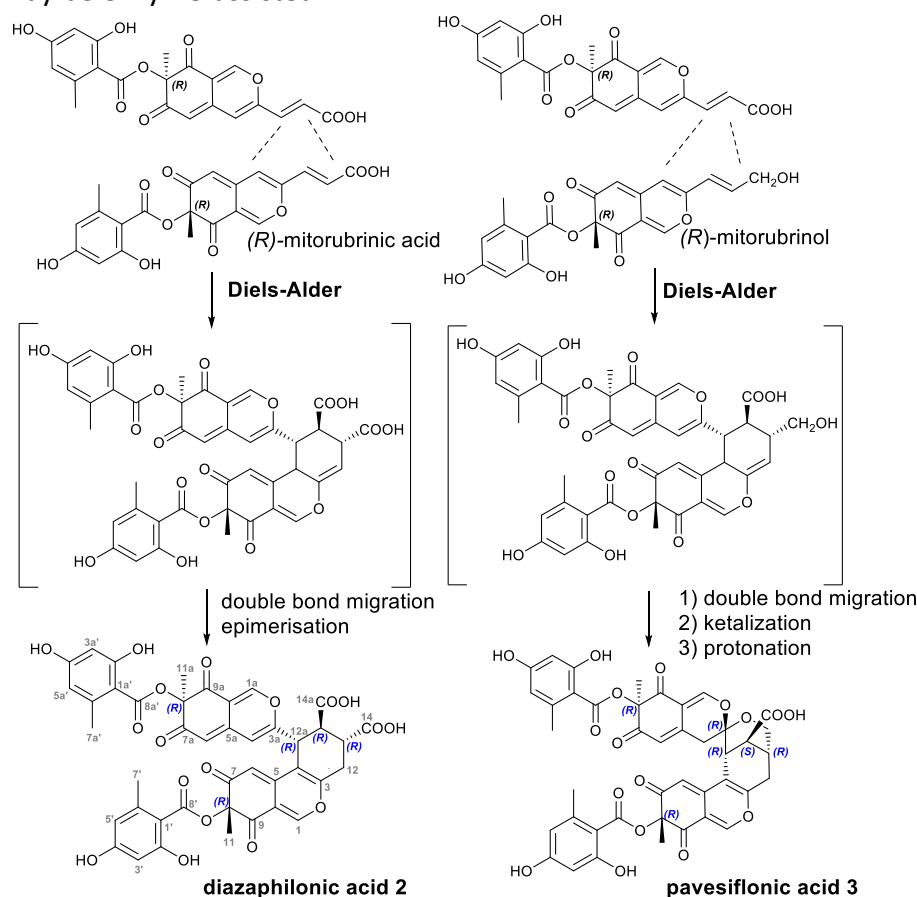


Figure 6. Diazaphilonic acid (2) and pavesiflonic acid (3)

MN, in comparison to haloseeker, allowed us to indicate the presence of tagged compounds while providing structural information about these compounds. This approach was therefore exploited to detect the presence of novel azaphilic compounds in the crude extract of *Bartalinia robillardoides*, an Ascomycota of the Sporocadaceaea family. This strain has been

scarcely studied so far, but some chloroazaphilones were already identified.<sup>11</sup> After addition of 1, probe-azaphilic NPs adducts were monitored by LC-MS2 and processed by GNPS. The resulting MN, combined with dereplication in the azaphilone database,<sup>10</sup> pointed out the presence of a probe-azaphilone adduct cluster containing two known compounds, the 8-11-didehydrochermesinone B18 and colletotrichone A19, together with an unknown tagged compound at  $m/z$ : 762.1901, corresponding to the new azaphilone 4 at  $m/z$ = 265.0263 (C<sub>13</sub>H<sub>9</sub>ClO<sub>4</sub>) (Figure 7).

Compound 4 was isolated as an orange oil. Its molecular formula was established as C<sub>13</sub>H<sub>9</sub>ClO<sub>4</sub> from its positive HR-ESI-MS  $m/z$ : 265.0263 [M+H]<sup>+</sup> (calcd. for C<sub>13</sub>H<sub>10</sub>ClO<sub>4</sub> 265.0262) and indicating 9 unsaturation degrees. The <sup>1</sup>H NMR data showed two methyl groups  $\delta$ H 1.63 (3H, s, C13), 2.25 (3H, d, J= 0.6 Hz, C14) and three olefinic protons  $\delta$ H 5.26 (1H, d, J=1.0 Hz, H6), 6.40 (1H, s, H4), 8.20 (1H, d, J=1.0 Hz, H1). The <sup>13</sup>C spectrum revealed two methyl carbons, one deshielded oxygen-connected sp<sup>3</sup> carbon, seven sp<sup>2</sup> carbons including one ketone and one ester, and three sp<sup>2</sup> methine carbons (Table S3). 1H-1H COSY showed a spin-spin coupling system consisting of H-1 and H-6 and another consisting of H4 and CH314. Key HMBC correlations between H-4 and C3, C6, C5 and C10, between H1 and C5 and C10, as well as between H-6 and C8, C10, and between CH313 and C8, C7 indicated the presence of an isochromene moiety characteristic of an azaphilone skeleton (Figure S26). The chemical shift of the two quaternary carbons C11 and C12 at  $\delta$ H 115.7 and 166.6 respectively, together with the molecular formula of 4 allowed us to place an ester at C12 and a chloride at C11. The additional key HMBC correlations between CH313 and C9, between H1 and C9 and C11 defined the  $\gamma$ -lactone of compound 4. The absolute configuration at C8 was unambiguously determined as R by X-Ray crystallography and small Flack (Flack  $x$  = 0.02).<sup>18</sup> Therefore, compound 4 was assigned as shown (Figure 7) and named chlorobartaphilone. Noteworthy, it is also the first azaphilone to carry a chlorine at C11. During the isolation progress, compound 5 was also isolated and characterized as a chloro-hydroxy-chermesinone A (5) according 1D and 2D NMR spectra (Figure S33). The configuration of 5 was deduced based on key NOESY correlations between CH317 and CH211 which placed them on the same side of the molecule. The configuration of C13 was assigned as S, based on the CH318 shift ( $\delta$ C 16.3,  $\delta$ H 1.09) in comparison with the NMR data of colletotrichones A–C20 and chermesinone B.<sup>19</sup> It is noteworthy that chloro-16-hydroxy-chermesinone A (5) failed to react with the probe, probably because of low electrophilicity.

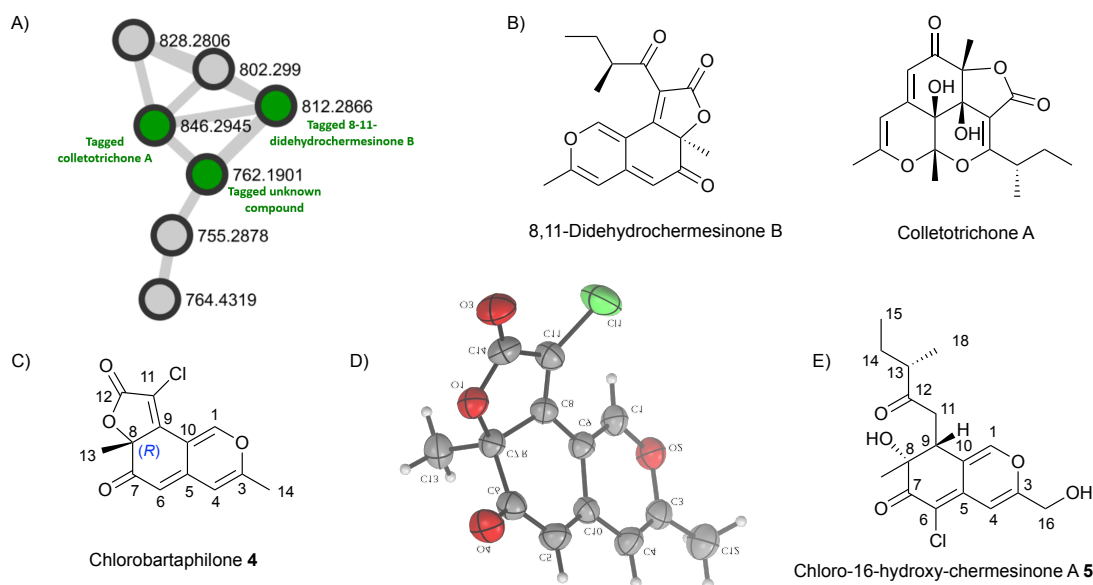


Figure 7. A) Cluster of tagged compounds from *B. robillardoides* obtained by molecular networking. Green nodes: compounds reacting with the probe and present in enough quantity to be isolated. B) known azaphilones isolated from *B. robillardoides*. C) Azaphilone (4) isolated from *B. robillardoides*. D) Ortep of Chlorobartaphilone (4). E) Azaphilone (5) isolated from *B. robillardoides*

This work highlights a bifunctional probe consisting of a nucleophilic primary amine for chemoselective reaction with azaphilic compounds and a brominated mass tag for MS detection. Exploiting this reactivity in combination with analytical tools for dereplication led to the detection, before isolation and structure elucidation, of novel fungal azaphilic compounds from complex crude extracts. Molecular Networks based on probe-NP adducts are powerful tools to cluster NPs of different families as they share common fragmentation pattern. Our findings thus open up exciting prospects for the rapid identification of azaphilic natural products from complex crude extracts and could be applied to robust and miniaturized screening of natural crude extracts in 96-well plates.

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#### Data Availability

The data supporting this article have been included as part of ESI. Crystallographic data for 4 has been deposited at the CCDC under CCDC 2218370 and can be obtained free of charge via [www.ccdc.cam.ac.uk/data\\_request/cif](http://www.ccdc.cam.ac.uk/data_request/cif).

#### Conflicts of interest

The authors declare no conflict of interest.

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