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Ljubica Svilar, Vesna Stankov-Jovanovic, Denis Lesage, H elo ise Dossmann, Jean-Claude Tabet. High-resolution mass spectrometry and hydrogen/deuterium exchange study of mitorubrin azaphilones and nitrogenized analogues. *Journal of Mass Spectrometry*, 2012, 47 (8), pp.969-977. 10.1002/jms.3044 . hal-04155785

**HAL Id: hal-04155785**

**<https://hal.science/hal-04155785v1>**

Submitted on 17 Jul 2023

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## **High resolution mass spectrometry and hydrogen/deuterium exchange study of mitorubrin azaphilones and nitrogenized analogues**

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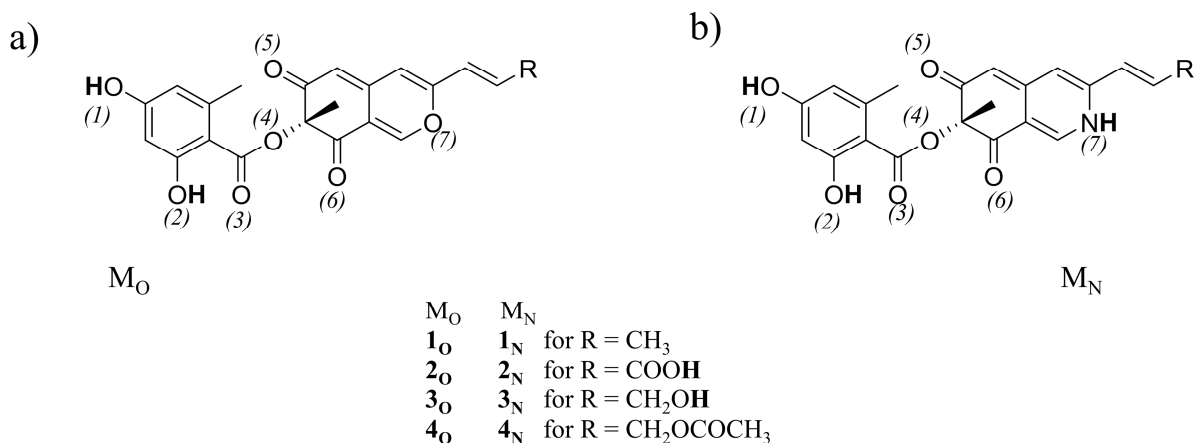
## Abstract

Azaphilones represent a numerous group of wild fungal secondary metabolites that exhibit exceptional tendency to bind to nitrogen atoms in various molecules, especially those containing amine group. Mitorubrin azaphilones are present in methanol extract of *Hypoxylon Fragiforme* fruiting body while their nitrogenized analogues have been detected in very low concentrations. Positive electrospray ionization (ESI) interfaced with high resolution mass spectrometry (FT/MS) was applied for confirmation of the elemental composition of protonated species. Collision induced dissociation (CID) experiments have been performed, and fragmentation mechanisms have been proposed. Additional information regarding the behaviour of both secondary metabolite analogue families has been reached by application of gas phase proton / deuterium (H/D) exchanges performed in the collision cell of a triple quadrupole mass spectrometer. An incomplete H/D exchange with one proton less than expected was observed for both protonated mitorubrin azaphilones and their nitrogenized analogues. By means of the density functional theory (DFT), an appropriate explanation of this behaviour was provided, and revealed some information concerning gas phase H/D exchange mechanism and protonation sites.

**Keywords:** mitorubrins, LC-ESI-FT/MS, CID, gas-phase H/D exchange, DFT

## Introduction

Azaphilones are secondary metabolites and natural fungal pigments which representatives have various structures and manifest number of biological activities such as antimicrobial, antibacterial, nematocidal, antimalarial, cytotoxic and antioxidant.<sup>[1,2,3,4]</sup> Their name (“nitrogen loving”) comes from their ability to react spontaneously with the amino groups from amino acids, proteins or nucleic acids to form nitrogenized analogues by the exchange of pyran-oxygen by a nitrogen atom. Among the different families of azaphilones, mitorubrin presents a class that can be often found in the *Hypoxylon Fragiforme* fungus and which is usually used as a fingerprint of these species.<sup>[5,6,7]</sup> The general structure of mitorubrins consists an orsellinic acid moiety attached to a bicyclic azaphilone core by an ester bond. The different members of the mitorubrin family differ by substituent R group located at the side chain end of the bicyclic core (Scheme 1a). By reacting with an amino group,<sup>[8]</sup> a nitrogenized mitorubrin analogue is formed,<sup>[9]</sup> (Scheme 1b).



**Scheme 1.** General structure of (a) mitorubrin M<sub>O</sub> and (b) nitrogenized mitorubrin derivatives M<sub>N</sub> (hydrogen atoms displayed with a bold letter are possibly exchangeable).

Various structural studies of mitorubrins can be found in the literature,<sup>[10,11]</sup> some of them use mass spectrometry techniques mostly *via* ESI and time of flight (TOF) or quadrupole mass analyzers.<sup>[12,13]</sup> Quite surprisingly, we were not able to find any work related to a structural characterization of nitrogenized analogues although different synthesis routes to form these compounds are described.<sup>[14,15]</sup> This is mostly due to the fact that these molecules exist only as traces in natural samples which thus avoid any further characterization with classical analytical methods. By studying extracts of some samples of *Hypoxylon Fragiforme* fungus, we recently found out that they contain two classes of compounds, one corresponding to mitorubrins and the other to nitrogenized analogues. To our knowledge, this is the first time that these nitrogenized analogues could be detected in that fungi species. We have thus decided to undertake a complete structural study of these compounds using FT/MS, coupled to high performance liquid chromatography (HPLC). The use of HPLC was justified as we were working with complex mixtures of mitorubrins and analogues.

Furthermore, in order to secure higher confidence data, we decided to perform additional analytical techniques. The studied molecules have been indeed represented in a very low concentration in fungi extract and were thus impossible to separate in adequate amounts for analysis. This has reduced number of reliable and powerful analytical methods such as various NMR and IR/UV-VIS spectroscopies by which, we could strengthen our first findings. The information that protonated nitrogenized analogues contain one exchangeable proton (see possible exchangeable hydrogen atoms at Scheme 1 a and b) more than the oxygenized azaphilones gave us idea to apply gas phase H/D exchange method.<sup>[16, 17]</sup> H/D exchange between selected gaseous ions and suitable deuterated agents is a well-established mass spectrometry method.<sup>[18]</sup> It is an ancillary and practical technique for determination of different compounds with functions containing mobile protons often bounded to heteroatom(s) such as alcohols, phenols, carboxylic acids, amines, amides and mercaptans, which are present in the molecular structures of natural organic compounds.<sup>[19,20]</sup> Gas phase H/D exchange rate constants depend mostly on the type of exchangeable protons (amine, amide, hydroxyl, etc.) and the used labeled reagent (i.e. the respective proton affinities of reagent and exchangeable site). Furthermore, the presence of intermolecular hydrogen bonds, steric effects

as well as other entropic effects can influence the gas phase H/D exchanges that can, for instance, occur with the relay mechanism.<sup>[16, 20]</sup>

In the present work we are showing a mass-spectrometric study of mitorubins and nitrogenized analogues originating from *Hypoxylon Fragiforme* fungus. This study includes the use of FT/MS coupled to HPLC as well as gas-phase H/D exchange reactions to validate our findings. Some DFT calculations have also been performed to help the understanding of the mechanisms involved in the course of the gas phase H/D exchanges.

## Experimental

*Hypoxylon Fragiforme* fungal samples were collected from the dead *Fagus Sylvatica* tree in the forest in South-Eastern Serbia and fungi material was deposited in the Herbarium Moesiacum, University of Nis (HMN), and voucher number 5530. Methanol and acetonitrile were of HPLC grade (VWR, EC). Water was obtained from a Milli-Q Gradient system (Millipore, Brussels, Belgium). Formic acid was from SDS (Carlo Erba Reactifs, France). ND<sub>3</sub> (99.75%) was obtained from Eurisotop (CEA Group, France). Standard mixtures used for the external calibration of the MS instrument, Calmix (caffeine, L-methionyl-arginyl-phenylalanyl-alanine acetate (MRFA)) and Ultramark 1621, were from Thermo Fisher Scientific (Les Ulis, France).

Samples of fungi were crushed with pestle and mortar. 10 mg of the obtained powder were extracted with methanol two times for 10 minutes in ultrasonic bath at room temperature with a five-minute centrifugation after each extraction. Collected supernatants were evaporated and purified by solid phase extraction (SPE) in aim to remove highly non polar components that could be further harmful to the HPLC column. They were then eluted *via* a Strata Phenomenex C18 cartridge with 2 mL acetonitrile:water, 40:60 (v/v). Final concentration is estimated to be up to 0.2 mg/mL.<sup>[21]</sup> The sample was finally analyzed by liquid chromatography coupled to ESI-mass spectrometry (LC/MS). To this end, 5  $\mu$ L of sample were injected into a reverse-phase C18 Symmetry (2.1 x 150. 5 $\mu$ m) column from Waters (Ireland). Separation of the different species was done in isocratic mode using acetonitrile:water, 1:1 (v/v) with 0.1 % formic acid at a flow rate 200  $\mu$ L/min.

Accurate mass measurements were performed on an LTQ Orbitrap XL from Thermofisher Scientific (San Jose, CA, USA),<sup>[21]</sup> with the external calibration of the instrument, which specification of accuracy is up to 5 ppm. Positive ionization mode was chosen because it brings more structural information than the negative one. Parameters for ESI were the following: ESI needle 4 kV, capillary voltage 20 V, capillary temperature 275°C, nebulizer gas N<sub>2</sub> 70 arbitrary units (a. u.), auxiliary gas 20 a. u. and tube lens offset 80 V. Mass spectra were recorded in the 145 – 900 Th range. CID experiments were done in the linear ion trap using helium as a collision gas, in the 100 - 500 Th range with 1 Th isolation width and 15 % of normalized collision energy<sup>22</sup>. The mass resolving power (full width at the half maximum height, FWHM) was set at 6 10<sup>4</sup> for signal at mass  $m/z$  400.<sup>[23]</sup>

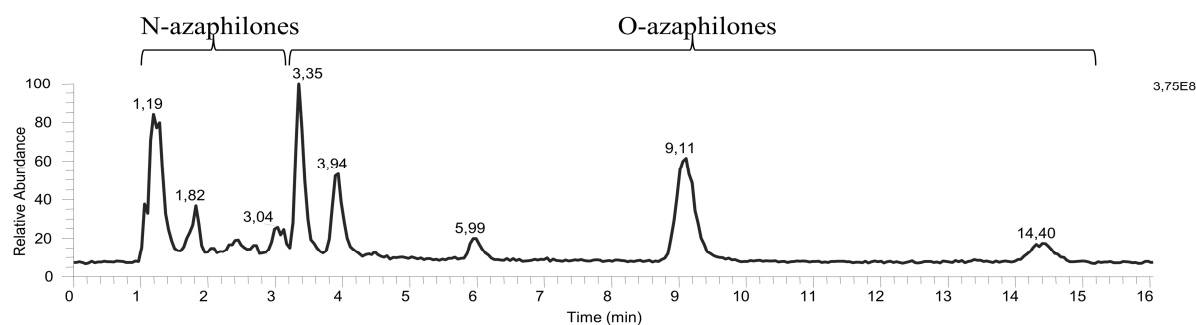
Gas phase H/D exchange reaction studies on azaphilone compounds were undertaken on modified triple quadrupole mass spectrometer Quattro II (Micromass, Manchester, U. K.). The data were acquired using Masslynx software (Version 4.2). The ESI capillary voltage was maintained at 3.5 kV. Compounds were ionized with a cone voltage of 30 V, and “in source-

CID” was performed with a cone voltage of 60 V. Nitrogen was used as the nebulization and desolvation gas. The source and desolvation temperatures were kept at 80°C and 20°C, respectively. ND<sub>3</sub> was introduced into the hexapolar collision cell at a pressure of 3.10<sup>-3</sup> mbar for collision-activated reactions (CAR) on selected precursor ion [24, 25]. CAR describes multiple collision ion-molecule reactions that occur in the RF-only quadrupole cell of triple quadrupole between selected ion beam (from the first quadrupole filter) and neutral reagent introduced *via* the Helium line at the higher pressure as possible. [26] These ion-molecule reactions occur at very low energy collision conditions for exothermic processes ( $E_{lab}$  of few eV) in contrast to CID. However, for slightly endothermic ion-molecule reactions larger ion kinetic energies are required without reaching the CID threshold. [27] Under these low energy collision conditions (i.e., herein as  $E_{lab} = 3$  eV, or  $E_{CM} \approx 0,15$  eV), multiple neutral/ion interactions occur yielding both the ion kinetic and internal energy relaxation, for precursor ion solvation which is an exothermic process, and resulting into relative long life ion-dipole complexes. Analyzed protonated molecules and in-source fragment ions were mass selected in the first quadrupole with isolation  $m/z$  width equal to 1 Th and then sent to a radiofrequency (rf)-only hexapolar collision cell where they were submitted to ion-molecule reactions with labeled reagent gas. The use of set of lens voltage 7, 8 and 9 (250, 0 and 0 V respectively) was crucial for successfully storing the selected precursor ions for the CAR experiments. The reaction product ions were mass analyzed by voltage scanning in the second quadrupole mass analyzer.

In parallel to experiments, quantum chemistry calculations have been performed using the Gaussian 09 program. [28] Geometry optimizations were obtained with the B3LYP functional [29,30] and the def2-SVP split valence basis set. [31] Single-point energies were obtained with the same method and the Def2-TZVPE basis set. Structures were characterized either as minima or transition structures from vibrational analysis. Proton affinities (PA) were calculated for different sites of the mitorubrin compounds using atomization energies. Results presented were corrected by a linear factor obtained by comparing the results with well-defined proton affinities. [32]

## Results and discussion

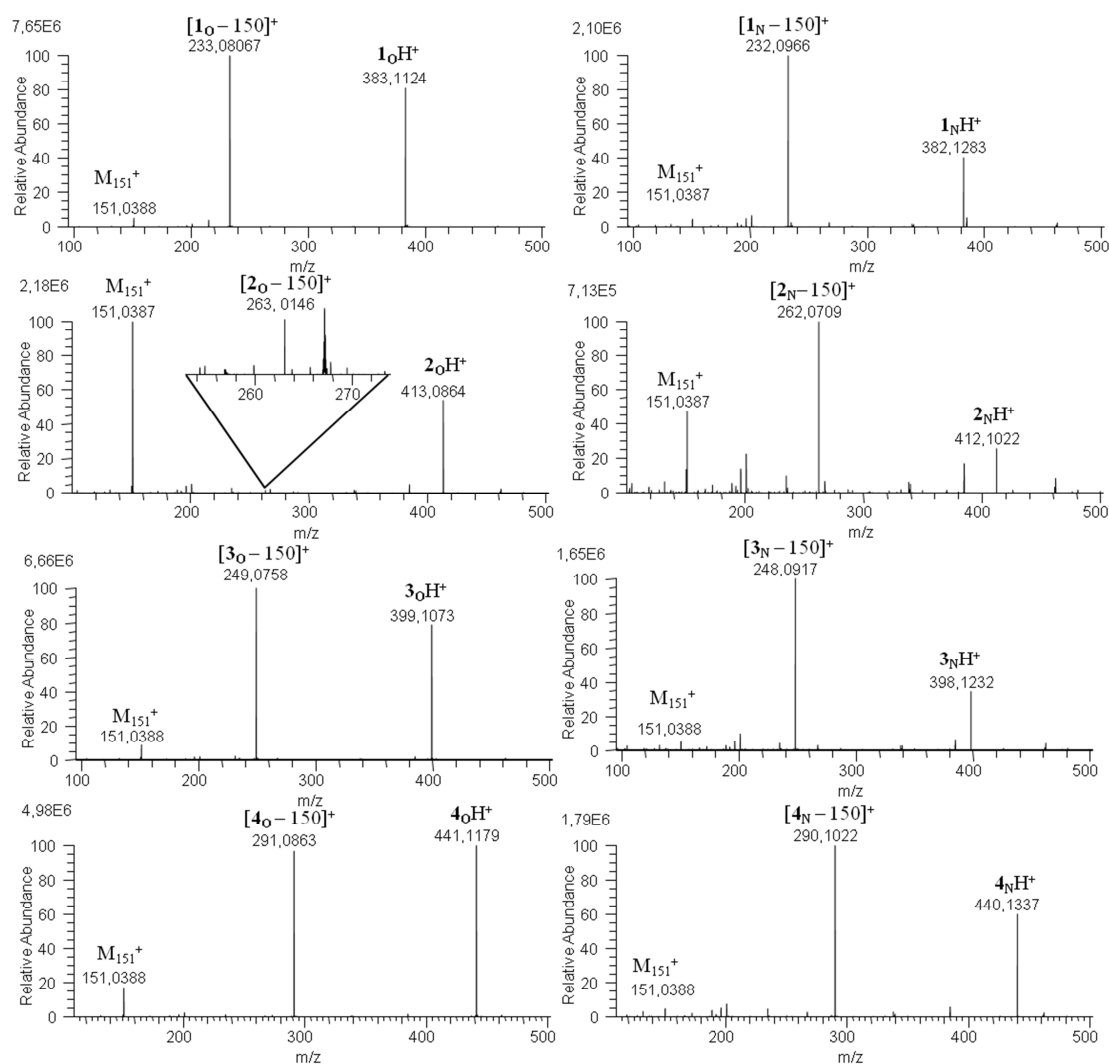
*Hypoxilon Fragiforme* methanol extract was at first separated using HPLC. Corresponding total ion current (TIC) is shown in Figure 1, and retention times and accurate elemental compositions are listed in Table 1.



**Figure 1.** Total ion current of the *Hypoxilon Fragiforme* methanol extract

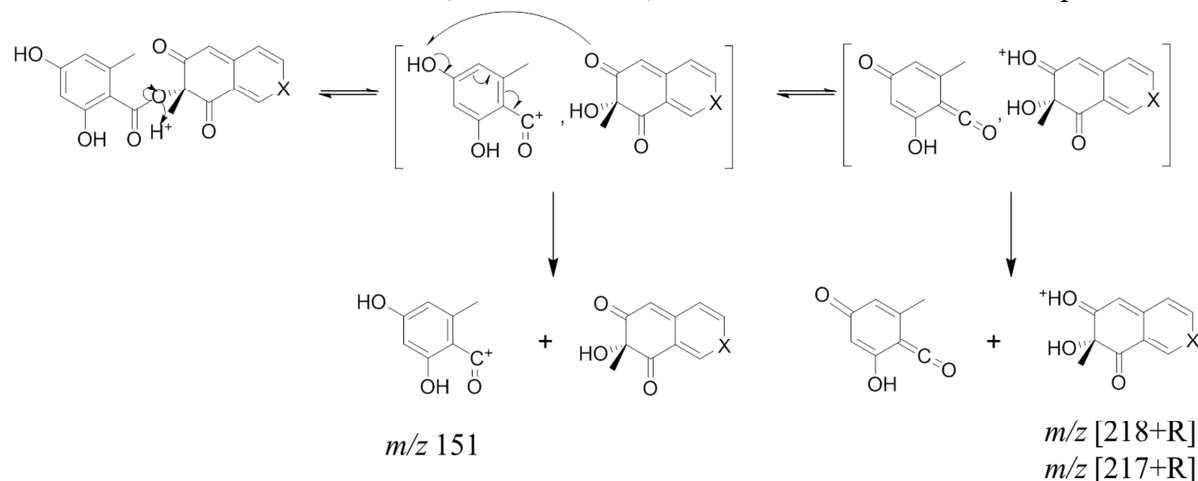
Each separated ion could further be identified *via* its elemental composition obtained by the high resolution of the instrument and given within 1 ppm of accuracy. In the first three and half minutes of chromatographic separation, ions with an elemental composition corresponding to nitrogenized analogues,  $M_N$  ( $1_N$ ,  $2_N$ ,  $3_N$  and  $4_N$ ; see Scheme 1) were observed whereas ions corresponding to mitorubrin azaphilones,  $M_O$  ( $1_O$ ,  $2_O$ ,  $3_O$  and  $4_O$ ; see Scheme 1) have a retention time larger than three minutes. A possible explanation for the nitrogenized analogues fast elution is their high polarity due to the presence of cyclic diene amine in comparison with cyclic diene ether group in mitorubrin.

The separated mitorubrins and their nitrogenized analogues have been then mass-selected and studied by the means of CID experiments of  $M_OH^+$  and  $M_NH^+$  ( $m/z$  [368+R] and  $m/z$  [367+R], respectively). Resulting CID spectra are shown in Figure 2.



**Figure 2.** CID (normalized collision energy 15%) spectra of protonated molecules of mitorubrins ( $M_O$ :  $1_OH^+$ ,  $2_OH^+$ ,  $3_OH^+$  and  $4_OH^+$ ) and nitrogenized derivatives ( $M_N$ :  $1_NH^+$ ,  $2_NH^+$ ,  $3_NH^+$  and  $4_NH^+$ )

For all compounds, a common  $m/z$  151 ion is observed. Its formation occurs by stepwise process *via* isomerization of protonated molecules into an ion-dipole complex<sup>[33]</sup>, prior to dissociation, as given in Scheme 2. Within this complex, proton transfer may occur, leading to the formation of the  $m/z$  [218+R] (or  $m/z$  [217+R]) ion, also observed in the CID spectra.

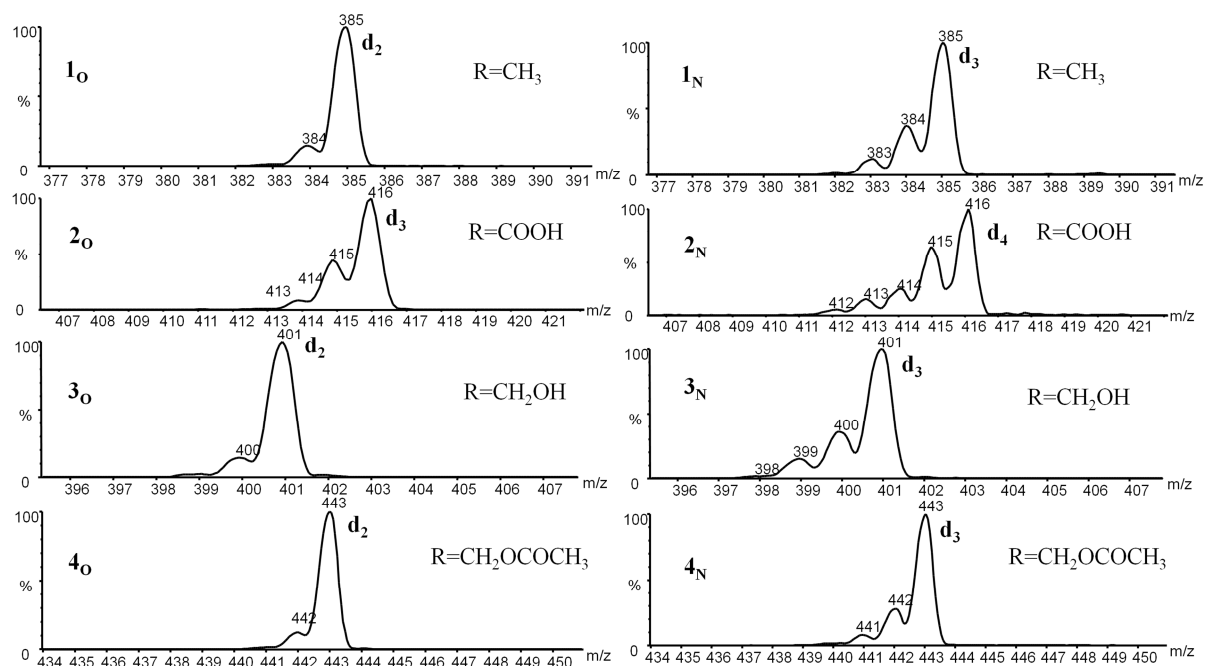


**Scheme 2.** Proposed mechanism for the observed dissociation of protonated azaphilones and nitrogenized azaphilones, *via* ion-dipole isomerization (R = CH<sub>3</sub>, CH<sub>2</sub>OCOCH<sub>3</sub>, COOH, CH<sub>2</sub>OH, and X = O, NH).

The relative abundances of the  $m/z$  151 and  $m/z$  [218+R] (or  $m/z$  [217+R] for nitrogenized analogues) ions vary accordingly to the side chain group. In general, nitrogenized analogues give a stronger fragmentation than protonated mitorubrin azaphilones. In the special case of mitorubrinic acid ( $m/z$  413), the abundance of  $m/z$  151 is higher than that of the complementary  $m/z$  [218+R] ion ( $m/z$  263). This can be explained by the mesomeric effect of carboxylic group of the side chain, that can diminish the proton affinity of the oxygen O<sub>(5)</sub> (see Scheme 1), and the formation of the common  $m/z$  151 ion is thus strongly favored. In case of mitorubrinic acid nitrogenized analogue ( $m/z$  412), this effect is decreased with the mesomeric effect assistance of nitrogen atom at N<sub>(7)</sub>, thus the complementary  $m/z$  262 and  $m/z$  151 fragment ions appear in comparable abundances.

Gas-phase H/D exchange reactions have further been performed in a collision cell of triple quadrupole instrument under very low collision energy conditions ( $E_{\text{lab}} = 3$  eV). Parent ions were isolated in the first mass filter of triple quadrupole and have undergone reactive collisions with a labeled reagent gas. ND<sub>3</sub> was chosen as a gas-phase labeled reagent because of its relatively high PA (204 kcal/mol),<sup>[32]</sup> thereby expecting exchange of the majority labile protons. The obtained CAR spectra are presented in Figure 3.



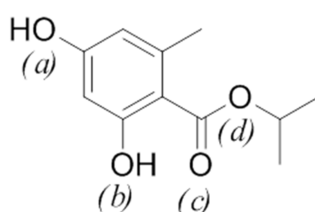


**Figure 3.** Gas phase H/D exchange CAR spectra ( $E_{\text{lab}} = 3 \text{ eV}$ ) of selected protonated mitorubrin ( $1_{\text{O}}\text{H}^+$ ,  $2_{\text{O}}\text{H}^+$ ,  $3_{\text{O}}\text{H}^+$ ,  $4_{\text{O}}\text{H}^+$ ), and corresponding protonated nitrogenized derivatives ( $1_{\text{N}}\text{H}^+$ ,  $2_{\text{N}}\text{H}^+$ ,  $3_{\text{N}}\text{H}^+$ ,  $4_{\text{N}}\text{H}^+$ )

Expected positions of exchangeable protons on protonated mitorubrin are (i) the one of protonation, thus  $\text{O}_{(4)}$ , (ii)  $\text{O}_{(1)}$  and  $\text{O}_{(2)}$  atoms and (iii) within the side chain, making in that way possibility at least three protons to be exchanged. Concerning nitrogenized mitorubrin analogues, four protons at least should be exchanged, as these compounds possess in addition, a secondary amine unit with an exchangeable proton. Unexpectedly, all precursor ions showed a number of exchanged protons lower by one, resulting in exchange of only two protons in protonated mitorubrin  $1_{\text{O}}\text{H}^+$ ,  $3_{\text{O}}\text{H}^+$  and  $4_{\text{O}}\text{H}^+$ , and three protons in  $2_{\text{O}}\text{H}^+$ . Furthermore, respective nitrogenized mitorubrin analogues exchanged one proton more than corresponding oxygenated derivatives.

Generally, ionizing proton is exchanged with a suitable labeled reagent. However, this proton can be spectator of the H/D exchange process, in the case of relay mechanism, where the exchange takes place from a labile proton distant to the former one (*vide infra*). In the present case, the H/D exchange at the  $\text{N}_{(7)}\text{H}$  site is somewhat unexpected because of its low PA value (Table 2), compared to that of ammonia. To explain this behavior, it can be considered the occurrence of electron lone-pair delocalization of the  $\text{N}_{(7)}$  atom, when the  $\text{O}_{(5)}$  atom is protonated. Under these conditions, withdrawing-electron effect takes place yielding electron delocalization from the distant  $\text{N}_{(7)}\text{H}$  site to  $\text{O}_{(5)}\text{H}$ . Consequently, it results a possibility of  $\text{ND}_3$  reagent to remove the former proton leading to formation of ion-dipole complex between  $\text{HND}_3^+$  and neutral. The structure of the latter one is tautomer to that of the initial nitrogen mitorubrin analogue molecular form. By this way, the  $\text{N}_{(7)}$  atom within the formed pyridine ring can receive deuteron from the  $\text{HDN}_3^+$  ions in the complex. By this way, H/D exchange occurs at the  $\text{N}_{(7)}\text{H}$  site (See supplementary Data Scheme S3).

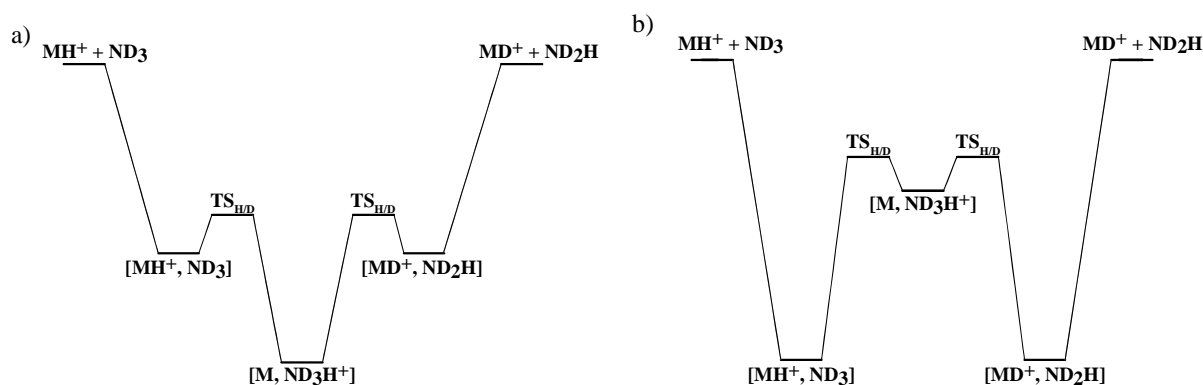
To understand these unexpected features, we have undertaken a theoretical study on a model system shown in Scheme 3. This system contains only the orsellinic part of the mitorubins, because there was no need to consider the bicyclic azaphilone core of the compounds, since gas phase H/D exchanges take place on this side of the molecule including either the R group, or the amine, in the case of the nitrogenized analogues. Hence, no protons are exchanged for R = CH<sub>3</sub> or CH<sub>2</sub>OCOCH<sub>3</sub> because these groups do not contain labile protons. For R = COOH, one proton was exchanged as expected. In case of R = CH<sub>2</sub>OH, one proton exchange could be assumed. However, in this case the mechanism would imply most likely, that protonation of **3**O<sup>H</sup><sup>+</sup> (or **3**N<sup>H</sup><sup>+</sup>) is carried by oxygen, leading immediately to a loss of water, favored by its allylic position. Thus, no proton is actually exchanged at this site, because fast water release occurs instead.



**Scheme 3.** Structure of the molecule **5** used as a model system for calculations.

In aim to achieve better insight in mechanism, the model system **5** has been chosen with an isopropyl ester form because it possesses similar PA values of its O<sub>(a)</sub>, O<sub>(c)</sub> and O<sub>(d)</sub> sites as on the O<sub>(1)</sub>, O<sub>(3)</sub> and O<sub>(4)</sub> sites of mitorubins and nitrogenized analogues (respectively 182.6, 225.1 and 215.7 kcal/mol to be compared with the PA values given in Table 2). It may be noticed that PA values were defined for each oxygen (and nitrogen) atom of the molecule, except for O<sub>(2)</sub> because calculations showed that protonation at this site leads to an prompt proton transfer to the O<sub>(3)</sub> atom.

Gas phase H/D exchange processes, have been extensively studied since now.<sup>[34, 17]</sup> The general mechanism of H/D exchange between an MH<sup>+</sup> ion and ND<sub>3</sub> is depicted in Scheme 4. Two cases can be distinguished, depending on the nature of the proton exchanged. If the PA of M is lower than that of ND<sub>3</sub>, than the solvated [MH<sup>+</sup>, ND<sub>3</sub>] form can isomerize into [M, NHD<sub>3</sub><sup>+</sup>], which is much more stable and can lead to formation of [MD<sup>+</sup>, NHD<sub>2</sub>] complex (Scheme 4a). The latter one gives labeled MD<sup>+</sup> and NHD<sub>3</sub><sup>+</sup> ions by competitive dissociation,. Reversely, when PA of M is higher than that of ND<sub>3</sub>, the TS energy is significantly increased and becomes close to the initial energetic level. This TS can be lowered by assistance (relay mechanism) [19] (Scheme 4b), meaning that the ionizing proton is not necessarily exchanged during this stepwise process (*vide supra*).

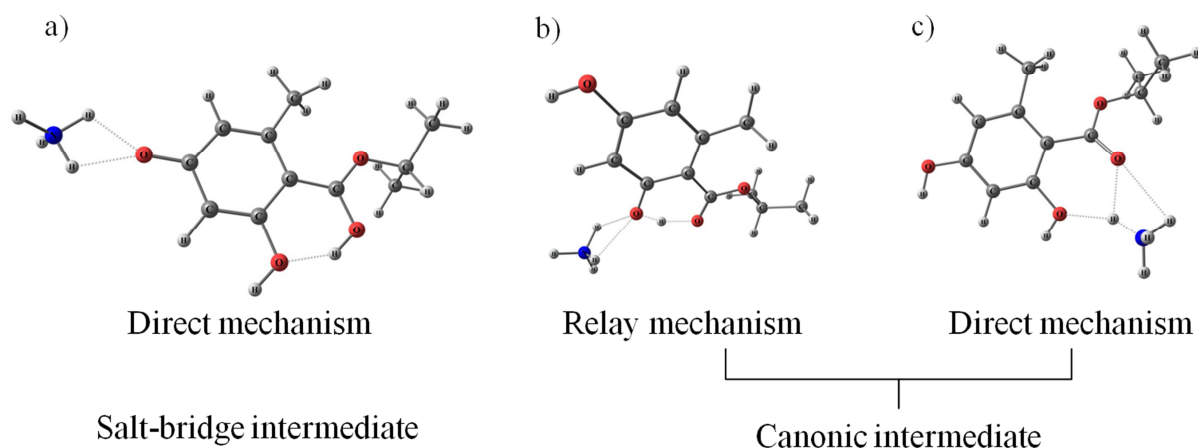


**Scheme 4.** General mechanism of an H/D exchange process between a  $\text{MH}^+$  ion and  $\text{ND}_3$ , in the case of exchange of (a)  $\text{PA}(\text{NH}_3) > \text{PA}(\text{M})$ , and (b)  $\text{PA}(\text{NH}_3) < \text{PA}(\text{M})$ .

The behavior of each protonated form describes the reaction of the  $[\text{5H}^+, \text{ND}_3]$  complex within a protonated canonic structure when (i) the ionizing proton is exchanged, or (ii) a relay mechanism takes place. However, zwitterion-like form could be involved into assumed canonic structure,  $[(\text{5-H})^-\text{H}^+, \text{NHD}_3^+]$ . In this case, the proton transferred to  $\text{ND}_3$  comes from the  $\text{O}_{(a)}\text{H}$  site to give rise to formation of salt-bridge complex, in which the ionizing proton at the ester site is spectator. From such ion-dipole complex, H/D exchange can take place in the salt-bridge position. This can occur only if the formation of the complex requires relative low TS energy. Since the calculated transition state with  $\text{O}_{(a)}$ , appears slightly endothermic (Table 3), previous considerations need to be disregarded, as well as assumed salt-bridge intermediate.

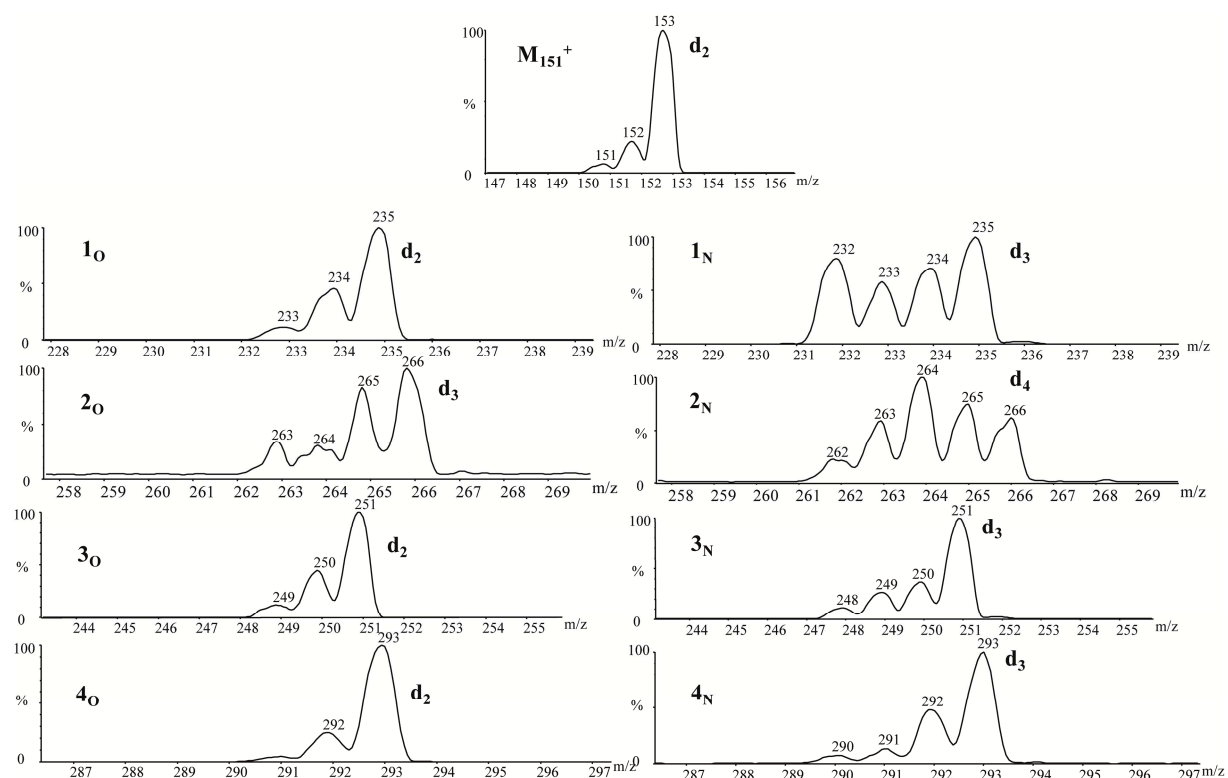
On the other hand, as protonation takes place during ESI, within the protonated canonic form, the H/D exchange is hindered because of very low PA value of the  $-\text{OH}$  site (184.7 kcal/mol) comparing to that of  $\text{ND}_3$ . The deuteron attachment to this position is thus high endothermic.

The results of theoretical calculations obtained for the gas phase H/D exchange reaction on the three possible oxygen atoms,  $\text{O}_{(a)}$ ,  $\text{O}_{(b)}$  and  $\text{O}_{(c)}$ , of the protonated  $\text{5H}^+$  molecule are presented in Table 3. For each reaction (which will be further annotated according to the mark of the oxygen atom, *i.e.* reaction a), b) or c)) energies are given in relation to the starting system ( $\text{5H}^+ + \text{ND}_3$ ). Formation of the ion-dipole  $[\text{5H}^+, \text{ND}_3]$  complex (an ion solvation step) allows isomerization into the  $[\text{5}, \text{NHD}_3^+]$  complex, which is an exothermic process, releasing more energy in the case of b) and c) pathways (up to 16.2 kcal/mol) in comparison to a) pathway (9.4 kcal/mol). The transition states energies,  $\text{TS}_{\text{H/D}}$ , (Figure 4), are located at -8.9 and -11.0 kcal/mol, under the initial energy of the system for the b) and c) paths, respectively, in contrast to +0.3 kcal/mol, for path a). Furthermore, as the barriers for the reactions b) and c) are crossed over,  $[\text{5}_\text{D}\text{H}^+, \text{ND}_2\text{H}]$  and  $[\text{5}_\text{H}\text{D}^+, \text{ND}_2\text{H}]$  complexes are formed competitively, releasing the exchanged  $\text{5}_\text{D}\text{H}^+$  and  $\text{5}_\text{H}\text{D}^+$ , respectively. Transition state of the reaction b) leads to the H/D exchange, because this process occurs with assistance of the ionizing proton interaction from the  $\text{O}_{(c)}$  site, through the hydrogen bond with the  $\text{O}_{(b)}$  site. This process is impossible in the case of the  $\text{O}_{(a)}$ . These considerations suggest that the exchange is favorable at  $\text{O}_{(b)}$  and  $\text{O}_{(c)}$  sites exclusively.



**Figure 4.** Transition states for the H/D exchanges between  $5\text{H}^+$  and  $\text{ND}_3$  occurring on the sites a)  $\text{O}_{(a)}$ , b)  $\text{O}_{(b)}$  and c)  $\text{O}_{(c)}$  of the model system **5**.

Complementary  $[\text{M}_\text{O}\text{H}-150]^+$  (or  $[\text{M}_\text{N}\text{H}-150]^+$ ), and common orsellinic part ( $m/z$  151) fragment ions formed in source have been studied in gas-phase H/D exchange experiments, under the same CAR condition used previously. Hence, they were isolated in the first mass filter of triple quadrupole and undergone the very low energy reactive collision with. The obtained CAR spectra are presented in Figure 5.



**Figure 5.** Gas phase H/D exchanges from CAR spectra ( $E_{\text{lab}} = 3$  eV) of fragment  $m/z$  151 and  $[\text{MH}-(150)]^+$  ions from protonated azaphilones and nitrogenized derivatives

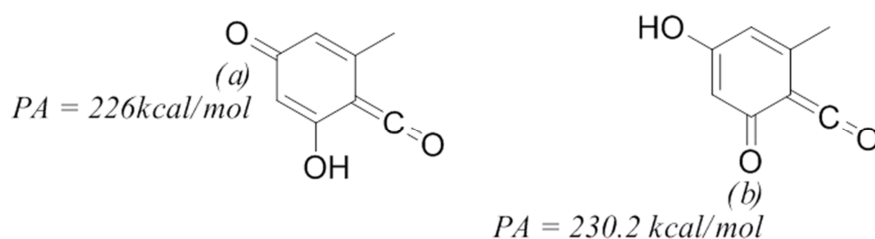
In the bicyclic fragment ion of mitorubrin azaphilones, at least two sites with labile protons are present, at the  $\text{O}_{(4)}$  and  $\text{O}_{(5)}$  positions, after the rearrangement during the fragmentation processes, whereas for the nitrogenized analogues there is also an  $\text{N}_{(7)}$  site with the labile

proton that could be exchanged. Thus,  $1_{\text{O}}\text{H}^+$ ,  $3_{\text{O}}\text{H}^+$  and  $4_{\text{O}}\text{H}^+$  exchange two protons by deuterons, while  $2_{\text{O}}\text{H}^+$  exchanges three. Concerning nitrogenized analogues, fragment ions of  $1_{\text{N}}\text{H}^+$ ,  $3_{\text{N}}\text{H}^+$  and  $4_{\text{N}}\text{H}^+$  exchange three protons by deuterium, while  $2_{\text{N}}\text{H}^+$  exchanges four. Hence, no side chain H/D exchange happens at  $\text{R} = \text{CH}_3$ ,  $\text{CH}_2\text{OH}$  and  $\text{CH}_2\text{OCOCH}_3$ , but for  $\text{R} = \text{COOH}$ , one H/D exchange takes place.

On the other hand, orsellinic part ion ( $m/z$  151) exchanges two protons by deuterium at the  $\text{O}_{(1)}$  and  $\text{O}_{(2)}$  positions, which can be referred to the  $\text{O}_{(a)}$  and  $\text{O}_{(b)}$  positions of the model system. This suggests that the protonated molecule isomerization into ion-dipole complex does not occur prior to CID processes. Otherwise, if it is the case, additional H/D exchanges could be expected through three body complex formation (Scheme 2).

Results of theoretical calculations obtained for the gas phase H/D exchange reaction of orsellinic acid fragment ion at  $m/z$  151, are presented at Table 4.

For both reactions named (a) and (b) for the  $\text{O}_{(a)}$  and  $\text{O}_{(b)}$  sites respectively, formation of ion-dipole complex [ $151^+$ ,  $\text{ND}_3$ ] is an exothermic process (-20.9 kcal/mol for (a) and -20.7 kcal/mol for (b)), in relation to the starting system ( $151^+ + \text{ND}_3$ ). The release of energy during the H/D exchange in (a) is almost the same as in the case of (b) (-20.9 kcal/mol for (a) and -20.7 kcal/mol for (b)), and the transition state for both paths are exothermic, -8.0 kcal/mol for (a) and -8.6 kcal/mol for (b). Relatively high transition state energy for this stepwise process is due to relatively high PA of neutral coming from  $m/z$  151, which is presented on the Scheme 5.



**Scheme 5.** Structures of neutrals from  $m/z$  151 and their PA

The PA of such *p*- and *o*-quinon-like neutral forms are higher than that of  $\text{ND}_3$ , meaning 226 kcal/mol and 230 kcal/mol, respectively, which is obtained by the calculations. In that way, final exchange at both sites leading to the H/D exchange on  $\text{O}_{(a)}$  and  $\text{O}_{(b)}$  could be explained, though, in both reactions, no isomerization in a second complex has been detected. This is probably due to the high stability of the complexes, which results in fast H/D exchange in the particular transition state, only achieved by calculations.

## Conclusion

Mitorubrin azaphilone secondary metabolites from the *Hypoxylon Fragiforme* fungus were characterized applying high resolution and gas phase H/D exchange mass spectrometry. Differentiation between the already known oxygenized derivatives and their nitrogenized

analogues was investigated. CID studies were performed and showed similarity between the ion structures as well as the fragmentation pathways for both families of mitorubrin analogues. Concerning the H/D experiments performed by CAR, one proton less was exchanged in all analyzed compounds. H/D exchange mechanism involves formation of ion-dipole complex, after the activation process. Resulted complex yields a product ion, in dependence of the PA values of both partners of the complex.

Theoretical studies of the gas-phase H/D exchange reaction mechanism have been applied to explain above mentioned phenomenon. In that way H/D exchange has evidenced as a powerful tool for structural distinction of both mitorubrin metabolite groups, oxygenated and nitrogenized. Calculations showed that a relay mechanism can take place from phenolic site that is adjacent to the ester group of mitorubrins or nitrogenized analogues. Finally, the low PA values of dienamine group can explain at least partially, why this proton was not exchanged.

### **Acknowledgments**

The authors want to thank Ministry of Education and Science Republic of Serbia (grant No 172051) and SM<sup>3</sup>P Platform for financial support.

We are thankful to Professor Mark Stadler (University of Bayreuth, Dept. Mycology, DNA Analytics and Ecoinformatics Germany) for determination of the fungi.

Ljubica Svilar expresses her sincere gratitude to the French Government for the scholarship provided for her Ph.D. program.

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