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Efficient biotransformation of non-steroid anti-inflammatory drugs by endophytic and epiphytic fungi from dried leaves of a medicinal plant, *Plantago lanceolata* L.

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Abstract

In the current study, decomposition of diclofenac, diflunisal, ibuprofen, mefenamic acid and piroxicam was tested using nine identified strains of endophytic and epiphytic fungi (from Ascomycota) adapted to natural products resembling the pharmaceuticals. The strains were isolated from a medicinal plant, *Plantago lanceolata* leaves. Metabolites were tentatively identified by liquid chromatography – tandem mass spectrometry (LC-MS^3).

Eighteen of the 45 combinations resulted in significant decrease of the concentration of the NSAIDs in model solutions. The most active strains were *Aspergillus nidulans* and *Bipolaris tetramera*, while *Epicoccum nigrum* and *Aspergillus niger* showed somewhat less potency. Piroxicam and diclofenac were most resistant to biotransformation, while ibuprofen and mefenamic acid were efficiently metabolized by most strains. Ten metabolites could be tentatively identified, including hydroxy-metabolites of all tested NSAIDs, and a dihydroxy-metabolite of piroxicam. This biotransformation is likely to modify the toxicity and bioaccumulation potential of these pharmaceuticals.

The results highlight the applicability of polyphenol-rich dried medicinal plant materials as an excellent source of fungi with high biotransforming potential. The results also suggest more in-depth testing of these fungi for biodegradation processes.

Keywords

biodegradation

biotransformation

non-steroid anti-inflammatory drugs

epiphytic fungi

phylloplane fungi

endophytes

Abbreviations

CE, capillary electrophoresis

DIC, diclofenac

DIF, diflunisal

IBU, ibuprofen

MEF, mefenamic acid

MEKC, micellar electrokinetic chromatography

NSAID, non-steroid anti-inflammatory drugs

PIR, piroxicam

1. Introduction

Non-steroid anti-inflammatory drugs (NSAIDs) are among the most frequently used drugs. These medicine are used to treat common conditions associated with many diseases, e. g. fever and pain (Hudec et al., 2008). The usage of high amounts results in appearance of these compounds and their metabolites in wastewater and also surface waters, as conventional wastewater treatment processes are unable to completely eliminate them (Domaradzka et al., 2015). Despite not being present at high concentrations enough to cause acute toxicity (they are typically detected in the ng l^{-1} to μg l^{-1} range (Paíga et al., 2015)), the risk of chronic toxicity can be significant (Domaradzka et al., 2015). For some pharmaceuticals, including the NSAIDs ibuprofen and naproxen, the toxic concentration against *Hydra attenuata* was found to be close to that detected in wastewater effluents (Quinn et al., 2008). Also, long-term exposure of fish to environmentally relevant concentrations of diclofenac is likely to cause impairment of the general health, and bioaccumulation (Schwaiger et al., 2004).

Significant research is ongoing to find microbial agents capable of decomposition of pharmaceuticals and other organic pollutants, like polyaromatic hydrocarbons (Harms et al., 2011). The fungi, like all organisms, enzymatically modify xenobiotics in order to decrease or eliminate their toxicity, if possible. The ability to completely decompose – mineralize – xenobiotics is not common during biodegradation, though there are several interesting examples (Harms et al., 2011). Hydroxylation, or other minor modifications, however, are more common. This step can be very important: it decreases the lipophilicity of the compounds, thereby reducing their bioaccumulation potential (Esser, 1986) and increasing their water solubility, and hence, bioavailability to enzymes for later degradation steps. It also reduces their ability to bind to solids which can be very significant for non-polar compounds, as shown for the NSAID mefenamic acid by (Jones et al., 2005). Also, hydroxylated compounds can have reduced toxicity compared to the original metabolite, as shown in the case of hydroxy-diclofenac metabolites in a yeast system (van Leeuwen et al., 2011). In other cases,

however, the hydroxy metabolites have been proven to be more toxic than the original compound. The metabolism of diclofenac (i.e. production of 5-hydroxy derivatives) correlated with toxicity in a hepatocyte model (Bort et al., 1999).

Several authors have described decomposition and/or transformation of NSAID drugs by fungi. Most of these studies used one of the so-called white-rot fungi (Domaradzka et al., 2015). Decomposition of a wide range of NSAIDs and other drugs was achieved for example by *Trametes versicolor* (Marco-Urrea et al., 2010; Rodríguez-Rodríguez et al., 2010; Tran et al., 2010). This species was also able to dechlorinate halogenated aromatics of high environmental persistence (Marco-Urrea et al., 2009a). Several studies have shown the oxidative modification of these drugs. Production of 4',5-dihydroxy-diclofenac, 4',-hydroxy-diclofenac and 5-hydroxy-diclofenac from the NSAID diclofenac by *Phanerochaete sordida* was reported by (Hata et al., 2010). The same fungus was shown to successfully metabolize mefenamic acid into several oxidized products, like 3-OH-mefenamic acid and 3-COOH-mefenamic acid (Hata et al., 2010). The ibuprofen metabolism also consisted of oxidation (hydroxylation) steps in the case of *Trametes versicolor*, but the final product (1,2-dihydroxy-ibuprofen) was found to be new, and not previously described in biological systems (Marco-Urrea et al., 2009b). Production of 4'-hydroxy-diclofenac by a non-white-rot fungal strain, *Epicoccum nigrum* was described by (Webster et al., 1998).

The endophytic and epiphytic fungi of medicinal plants represent another group of fungi: they are not involved in wood decay as the white-rot fungi, but they also live in a special chemical environment. They are surrounded by plant molecules, among which phenolic and other aromatic substances are especially common. The adaptation to this chemically unique habitat makes these organisms a rich potential source of biocatalysts (Suryanarayanan et al., 2012).

In the current study, we aimed to assess the potential of a cosmopolitan plant rich in phenolic compounds, dried leaves of *Plantago lanceolata* L., as a source of fungi useful for the biotransformation of NSAIDs. The tested strains included endophytic fungal lines as well as ubiquitous

fungi isolated from the plant material. These strains have been previously found to be able to produce a wide array of hydroxylated phenolics from the plant material, as well as decomposition of polyphenolic compounds (Gonda et al., 2013a). We aimed to test these nine identified strains for their NSAID decomposition ability in model solutions. The NSAIDs included piroxicam and diflunisal for which no fungal transformation data were available. We also aimed to gain structural information on the major NSAID biotransformation products by LC-MS/MS.

2. Materials and Methods

2.1. Chemicals. The following chemicals and reagents used were of at least analytical grade: diclofenac sodium (DIC, Sigma-Aldrich, St.Louis, MO, USA), diflunisal (DIF, Sigma-Aldrich), ibuprofen (IBU, Sigma-Aldrich), mefenamic acid (MEF, Sigma-Aldrich) and piroxicam (PIR, Sigma-Aldrich), saccharose (VWR, Budapest, Hungary), sodium carbonate (Reanal, Budapest, Hungary), sodium nitrate (Reanal), dipotassium hydrogenphosphate (Reanal), magnesium sulfate heptahydrate (Reanal), potassium chloride (Reanal), ferric sulfate heptahydrate (Reanal), 1-Propanol (VWR). For chemical structures of the NSAIDs, see Fig. 1. The pH of media was adjusted with sodium hydroxide (Reanal) or hydrochloric acid (Reanal). For LC-MS applications, water (0.055 μS/cm, produced by Sartorius Arium 611), methanol (Merck), and ammonium acetate (Merck) were of LC-MS grade. For other applications, bidistilled water was used. Ten mg mL^{-1} Stock solutions of NSAIDs were prepared in equimolar sodium carbonate solutions, filtered sterile and were stored at -24 ºC before use.

2.2. Fungal strains

Nine of the strains identified in one of our previous studies (Gonda et al., 2013a) were tested for NSAID decomposition abilities. The following strains were used: ***1***: *Aspergillus niger*; ***2***: *Eurotium*

repens; ***3***: *Leptosphaerulina chartatum*; ***4***: *Aspergillus nidulans*; ***5***: *Eurotium amstelodami*; ***6***: *Cladosporium pseudocladosporioides*; ***7***: *Penicillium chrysogenum*; ***8***: *Bipolaris tetramera*; ***9***: *Epicoccum nigrum*. The strains were isolated from a medicinal plant material rich in phenolic compounds. The isolation method resulted in a mixture of endophytes and epiphytic fungi. The rationale for testing these strains for NSAID biotransformation was the similarity of the NSAIDs and the phenolic compounds released by these strains from the plant material. The isolation method, the identification of the species and the gene accession numbers are described in (Gonda et al., 2013a).

2.3. Activity screening for non-steroid anti-inflammatory drug decomposition by plant-associated fungi.

Sterile, 10 mL aliquots of Czapek-Dox liquid media (CzD, 3.0 g $NaNO_3$; 1.0 g K_2HPO_4; 0.5 g $MgSO_4.7H_2O$; 0.5 g KCl and 0.01 g $FeSO_4.7H_2O$ per liter, supplemented with 2% saccharose, pH 7.3±0.2) were spiked with stock solutions of the five NSAIDs (resulting in 100 μg mL^{-1} end concentration for each) and inoculated with the different fungi - ¼ of the surface of a Czapek Dox Petri Dish fully covered with mycelia was scraped off, and suspended into the liquid medium. Control was not inoculated. After mixing, the inoculated solution was separated into 250 μL aliquots and incubated in a sterile environment for 28 days. Evaporation was prevented by the saturation of the surrounding air with water vapor. After the end of incubation time, the aliquots were lyophilized, dissolved in 40 mM disodium phosphate containing 10% 1-PrOH, centrifuged at 13,000 rpm for 5 minutes and analyzed by capillary electrophoresis micellar electrokinetic chromatography (CE-MEKC) directly for residual NSAID amounts.

CE measurements were carried out on a PrinCE-C 700 capillary electrophoresis instrument with a 60 cm fused silica capillary with 50 m i.d. Effective length was 52.8 cm. Capillary preconditioning and post-conditioning were previously described (Gonda et al., 2013b). Sample injection was

hydrodynamic (100 mbar × 0.10 min.), NSAIDs were quantified at 200 nm. Background electrolyte consisted of 40 mM disodium phosphate, 100 mM SDS, 3% MeOH, pH was set to 8.0. The method is adapted from (Maboundou et al., 1994) with a few modifications. Calibration curves contained mixtures of the five NSAIDs in 40 mM disodium phosphate containing 10% 1-PrOH. Concentrations of 25, 50, 75, 100, 125 and 150 μg mL^{-1} were used.

2.4. Identification of NSAID metabolites by LC-ESI-MS/MS

Fourteen fungus-NSAID pairs were selected for the study of NSAID metabolites. DIF was tested for decomposition by *A. niger* and *A. nidulans*; DIC by *A. nidulans* and *B. tetramera*; IBU and MEF by *A. niger, A. nidulans, B. tetramera* and *E. nigrum*; PIR by *A. niger* and *A. nidulans*. 5.00 mL of CzD media were spiked with a single NSAID of interest (100 μg mL^{-1} end concentration) and inoculated with different fungi. All incubation samples (with the same conditions as in 2.3.) had two controls: the fungus without any NSAID, and the NSAID in the medium without the fungus. After the 24 days' incubation time elapsed, samples were lyophilized. To the lyophilized samples, 500 μL of water was added until most of the pellet redissolved. Thereafter, 4.50 mL of MeOH was added, the samples were mixed thoroughly, sonicated for a minute and centrifuged (13000 rpm, 1 minute) to remove the mycelia. The supernatant was used for LC-MS analysis directly, using the following conditions: LC was performed on a Kinetex XB-C_{18}, (100 x 2.10mm, 2.6μm, Phenomenex, Torrance, CA, USA) column, using an Accela HPLC system (Thermo Electron Corp., San Jose, CA, USA) eluted with a gradient of methanol (A) and water (B) containing 0.1% NH_4OAc each. The gradient was from 10% of A to 100% A over 15 min, held for 5 minutes, returned to initial conditions in 0.10 minutes and held for 3 min to equilibrate the column. The LC system was coupled to a Thermo LTQ XL linear ion trap mass spectrometer (Thermo Electron Corp., San Jose, CA, USA) operated in a full scan positive or negative ion ESI mode (*m/z* range was 150-2000 Da for the main experiments). The ion injection time

was set to 100 ms. ESI parameters were: source temperature 275 °C; spray voltage 4 kV; capillary temperature 275°C; sheath gas flow 15 units N_2; auxiliary gas flow 8 units N_2. The tray temperature was set to 20°C and the column oven was set to 30 °C to perform the optimal retention of the compounds in the samples. The injection amount was 1 µL for each sample.

The peaks (*m/z* at retention time) found in LC-MS, which 1., were present in the medium of the fungus with NSAID (at least $5x10^2$ peak height); 2., were not present in the fungal metabolome control (fungus in medium without NSAID); 3., were not present in the noninoculated medium incubated with NSAID, were considered fungal NSAID metabolites. The peak list obtained was subjected to fragmentation in ESI-MS/MS in another run, to obtain structural information. The chromatographic conditions were the same.

2.5. Statistical evaluations and software.

LC-MS data evaluation was done with mzMine 2.10 (Pluskal et al., 2010) and the R package XCMS 1.42.0 (Smith et al., 2006). Data analysis was aided by using scripts written in R 3.1.2 (R Development Core Team, 2009), for plotting, ggplot2 was used (Wickham, 2009). ANOVA models were implemented in R and were used to statistically test hypotheses, followed by Dunnett's post-hoc test, where appropriate. The *p* values were considered significant when $p < 0.05$. Marvin 15.2.9.0-master-2515, 2015, ChemAxon (http://www.chemaxon.com), was used for drawing, displaying and characterizing chemical structures.

3. Results

3.1. Decomposition of NSAIDs by plant-associated fungi

The growth of the tested fungi was not inhibited by the added high amount of the NSAID mixture (sum of 500 µg mL^{-1}). The same strains also tolerated the high amount of polyphenolic

substances well in our previous study (Gonda et al., 2013a). In controls, no significant decrease of any of the NSAIDs was observed (**Fig.2.**), meaning, that spontaneous (hydrolytic or oxidative) decomposition did not occur during the experiments.

The fungal inoculation, however, significantly decreased the NSAID amount in the media after 28 days of incubation (p = 1.38e-15, 0.00526, 4.83e-10, 6.96e-16 and 5.21e-10 for IBU, PIR, MEF, DIF and DIC, respectively; n=3, ANOVA). The species showed marked differences in the ability to decrease the amount of NSAIDs in solution, and the sensitivity of the NSAIDs to enzymatic modification was also quite different. The most active strain was *Aspergillus nidulans*, which was the only strain that could decrease the amount of all NSAIDs in the test. It also caused complete biotransformation of the 100 μg mL^{-1} IBU by the end of the incubation time (p<0.001, **Fig.2.c.**). *A. niger* and the endophytic *B. tetramera* could biotransform three of the five NSAIDs: IBU, MEF and DIF (**Fig.2.**). The typical endophyte *E. nigrum* could metabolize two NSAIDs: IBU and MEF (p<0.05). Several of the strains, however, could only metabolize IBU (p<0.05, **Fig.2.c.**).

As it can be seen, the NSAIDs without any metabolism-resistant groups, i.e. IBU, MEF could be well metabolized by most strains (**Fig.2.c.,d.**). On the other hand, the halogenated aromatics (DIC and DIF), as well as the cyclic sulfonamide PIR were much less prone to decomposition (**Fig.2.a.,b.,e.**).

3.2. Metabolites of NSAIDs.

After successful screening for activity, a selected subset of fungi (*A. niger, A. nidulans, B. tetramera* and *E. nigrum*) were inoculated again with different NSAIDs separately for metabolite screening. As controls, the NSAID containing media alone, and the media of fungi without NSAIDs were used. Therefore, degradation products that might arise from spontaneous oxidative or hydrolytic decomposition, or compounds that can also be parts of the fungal metabolome, were excluded from the analysis.

To find possible metabolites, samples were grouped into sets consisting of fungus –

fungus+NSAID – NSAID, and subjected to automatic peak search, using parameters as in 2.4. This resulted in a preliminary peak set of 57 peaks. Another set was obtained by targeted screening for mono-, dihydroxy-compounds, and compounds that contained carboxyl groups instead of a methyl moiety. After removing the adducts, isotopes, as well as high *m/z* peaks found by the automatic search unlikely to be NSAID metabolites, the samples were reinjected and the selected list of peaks was fragmented in LC-MS^3. Identification was started in negative ion mode, with interesting peaks (i.e. peaks falling in the estimated retention time range for NSAID metabolites, and having a similar *m/z* to the initial molecules) rechecked in positive ion mode under the same chromatographic conditions. The compounds that could be identified as NSAID metabolites, are shown in **Table 1**. Several other peaks likely to be metabolites of the NSAIDs could be detected, but unfortunately, their abundance was insufficient to subject them to MS^3 to obtain structural information. Therefore, these are not dealt with further. Reduction of the concentration of the parent NSAIDs was reconfirmed by the LC-MS analysis.

Metabolism of DIC was only achieved by *A. nidulans* and led to three new peaks corresponding to DIC metabolites: two with *m/z* 310 ($[M-H]^-$) at 8.62 and 9.15 min., and one with *m/z* 150 at 4.32 min. The former two showed the typical isotope pattern of dichloro-compounds and showed very similar fragmentation patterns (**Table 1.**). These were identified as hydroxy-DIC derivatives as compared with the MS/MS data (Webster et al., 1998; Kallio et al., 2010). Although the actual position of the hydroxyl group could not be determined from the MS/MS alone, the fragmentation fully supported the proposed structure. The *m/z* 150 at 4.32 min is the result of the cleavage of the aromatic amine, the fungus successfully removed the dichloro-aromatic moiety from DIC. It can be observed, that the dehalogenation of the chlorinated aromatic group did not occur during the metabolism, i.e. no monochlorine aromatic breakdown product was found. The detected high abundance of the new metabolites account for the decrease of DIC in solution. The dominant metabolite was a OH-DIC isomer at 8.62 min, with a high abundance only an order of magnitude less than that of DIC. Assuming similar sensitivity for these two, the metabolite was likely to be present in the 1-10 $\mu g\ mL^{-1}$ range in the

medium. This suggests that after further optimization, *A. nidulans* could also be used for selective transformation of DIC.

A metabolite of DIF was identified in the medium of *A. nidulans*. The compounds was found to be a hydroxy-DIF metabolite, with the exact position of the -OH group not assigned. So far, no fungal metabolites of DIF have been described in the literature. The compound showed peaks identical to those described in the literature (Macdonald et al., 1991; Dickinson et al., 1994). As in the case of DIC, the halogenated ring was untouched; MS^3 provided evidence that the -OH is present on the salicylic acid moiety (*m/z* 153).

Metabolism of IBU by *A. niger*, *A. nidulans* and *B. tetramera* resulted in an intense new metabolite at 6.41 min ($[M-H]^-$ *m/z* 221). Fragmentation showed, that the metabolite is a hydroxy-derivative of IBU (Paíga et al., 2015), most probably the isobutyl sidechain is hydroxylated. The found fragmentation pathway was similar to that described for alkyl-phenols in (Bajpai et al., 2005).

Metabolism of MEF by *A. niger, A. nidulans, B. tetramera* and *E. nigrum* led to new peaks with the *m/z* 256 at 8.18 and 8.55 minutes. While the typically endophytic *Epicoccum nigrum* and strain *B. tetramera* created huge amounts of the first metabolite (rt 8.1 min), *A. nidulans* made significantly smaller amounts, but both peaks were observed. In the medium of *A. niger*, the amount of this metabolite was much less, but still, detectable. These compounds were identified as hydroxy-MEF isomers, as suggested by the fragmentation pattern in negative ion mode (Wing Lam et al., 2007).

Surprisingly, PIR was the most resistant NSAID of the ones tested. Partial metabolism of PIR could only be achieved by strain *A. nidulans*. So far, no fungal metabolites of PIR have been described in the literature. A major peak with *m/z* 346 $[M-H]^-$ at 7.14 min was detected as a new peak in the medium. This compound was identified as hydroxy-PIR, based on the fragmentation pattern (McKinney et al., 2004; Tevell Åberg et al., 2009). The fragmentation clearly indicated, that the pyridine ring was hydroxylated. Based on the fragmentation pathways described for meloxicam by (Tevell Åberg et al., 2009) two other peaks could be identified to be OH_2-piroxicam from the medium

of *A. nidulans* (*m/z* 362 $[M-H]^-$). The structures were supported by the fragmentation in both negative and positive ion mode. In this case, both the pyridine side chain and the sulfonamide part was found to be hydroxylated.

4. Discussion

Altogether, in 18 of the 45 cases has significant decrease been found, which highlights the potential of plant-associated fungi from medicinal plant materials for biodegradation of NSAIDs. It should be noted, that the fungi were isolated from the plant material with a procedure that leads to the mixture of endophytic and epiphytic fungi. Despite this, the adaptation to the phenolic compounds was evident, as shown previously by the ability to metabolise phenolics (Gonda et al., 2013a).

Decomposition of NSAIDs by fungi is previously described for a few of the tested molecules in the literature. The current study has shown, that the concentration of diflunisal and piroxicam can also be reduced by fungal treatment. Most of the studies used very low concentrations, and also, most studies used white-rot fungi. For example, the white-rot fungus *Phanerochaete chrysosporium* was able to metabolize 5 μg mL-1 DIC by its lignin peroxidase (Zhang and Geißen, 2010). Another white-rot fungus, *Trametes versicolor* successfully transformed higher concentrations (10 μg mL-1) of DIC into 4'-OH-DIC and 5-OH-DIC (Marco-Urrea et al., 2010). Our experimental setup shows the high tolerance of these fungi to the presence of high concentrations of NSAIDs (100 μg mL^{-1} order of magnitude), and the potency of fungi other than wood decay fungi in pharmaceutical biotransformation. It is also important to emphasize, that the efficacy is likely due to the chemical similarity of the NSAIDs and the small phenolic metabolites the strains are adapted to.

As decrease in the original molecule's concentration does not mean full decomposition of the compounds, a subset of fungi – NSAID combinations were screened for NSAID metabolites by LC-ESI-MS/MS. Altogether, ten metabolites were tentatively identified from the media of different wild-type epiphytic and endophytic fungi. Nine of these metabolites were shown to be either mono-, or

dihydroxy derivatives of the original anti-inflammatory drug molecules. The products generated by the fungi of the current study are similar to those found in the phase I metabolism of mammals - metabolism to various hydroxylated products was shown for these pharmaceuticals (Macdonald et al., 1991; Wing Lam et al., 2007; Stülten et al., 2008; Tevell Åberg et al., 2009; Borges et al., 2010). These metabolites can be less toxic or more toxic compared to the original compounds. In a study using an ex vivo intestinal model, metabolism inhibitors increased DIC toxicity, and also reduced the generated amount of 4'-OH-DIC, 5-OH-DIC (Niu et al., 2015). Oxydative biotransformation of DIC and MEF also reduced the toxicity against a freshwater crustacean in (Hata et al., 2010). In contrast, during the metabolism of IBU into 1,2-dihydroxy-IBU by *Trametes versicolor*, increase in toxicity was observed in Microtox bioassay (Marco-Urrea et al., 2009b). The oxidized NSAIDs (as shown for DIC) can also give rise to toxic adduct-forming reactive species when metabolized further (Tang et al., 1999). It is also important to note however that as oxidative modification increases the polarity of the compounds, their bioaccumulation is likely to be reduced, and their accessibility by enzymes is increased (Esser, 1986) helping later mineralization. Altogether, the change of toxicity of the NSAIDs by fungal metabolism is hard to predict without detailed toxicological studies on the metabolites themselves.

In a few cases, no metabolite could be detected by LC-MS, but the decrease in the solution was significant. This could have been the result of either metabolites that show low ionization and poor detectability by MS, or perhaps in a few cases, complete degradation.

The high amount of detected metabolites and the high chance of finding highly active fungi in such a small set of species clearly shows the potency of dried medicinal plant materials as sources of fungi useful for biotransformation. It is also worth to mention, that *B. tetramera* and *E. nigrum* were previously (Gonda et al., 2013a) shown to efficiently decompose the plant phenolic compound acteoside, and that many of these strains (including *A. nidulans* and *B. tetramera*) were able to release high amounts of small, oxidized phenolic compounds from the plant metabolome (Gonda et al., 2013a). Thus, the high chance of finding fungi that are able to oxidize NSAIDs was at least in part due to the

adaptation to biotransformation of small phenolic compounds of similar polarity highly abundant in the plant. It is also worth to mention, that the most easily metabolized IBU (8 of 9 strains caused significant decomposition) is the most similar to these phenolic natural products.

Dried medicinal plant materials can contain very significant amounts of spores of possibly adapted fungi, and also suitable nutrients for these fungi. This is especially true for products that are improper for human use because of their poor microbiological quality – these can contain up to 10^7-10^8 fungi per g dry weight (Gonda et al., 2012). Therefore, these materials should be tested in more practice oriented approaches for degradation or biotransformation of pharmaceuticals in the future.

5. Conclusions

The results of the current study suggest that dried medicinal plant materials can be excellent sources of fungi with high catalytic activity, regardless of the fungi being endophytic or epiphytic. The evolutionary adaptation of these fungi towards metabolism of bioactive natural products makes these strains tolerant to high concentrations of pharmaceuticals of similar structure, and applicable for transforming or biodegrading these pharmaceuticals. The biotransformation steps were found to resemble those of phase I mammalian metabolism. The wide availability of the tested source material can also make possible applications easily testable. The results also suggest, that fungi other than white-rot fungi should be investigated more in-depth for biodegradation processes.

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Figures

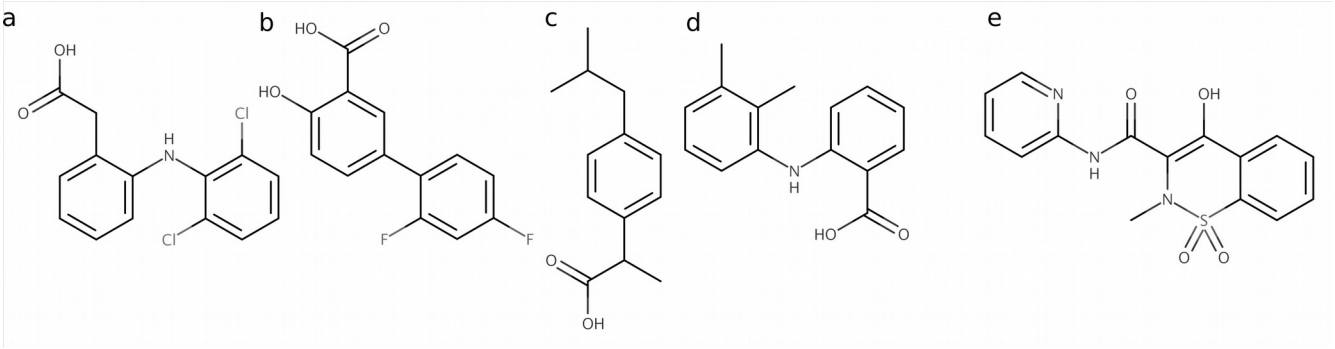


Fig.1. Chemical structures of the NSAIDs subjected to decomposition by plant-associated fungi. From left to right: a., diclofenac, b., diflunisal, c., ibuprofen, d., mefenamic acid, e., piroxicam.

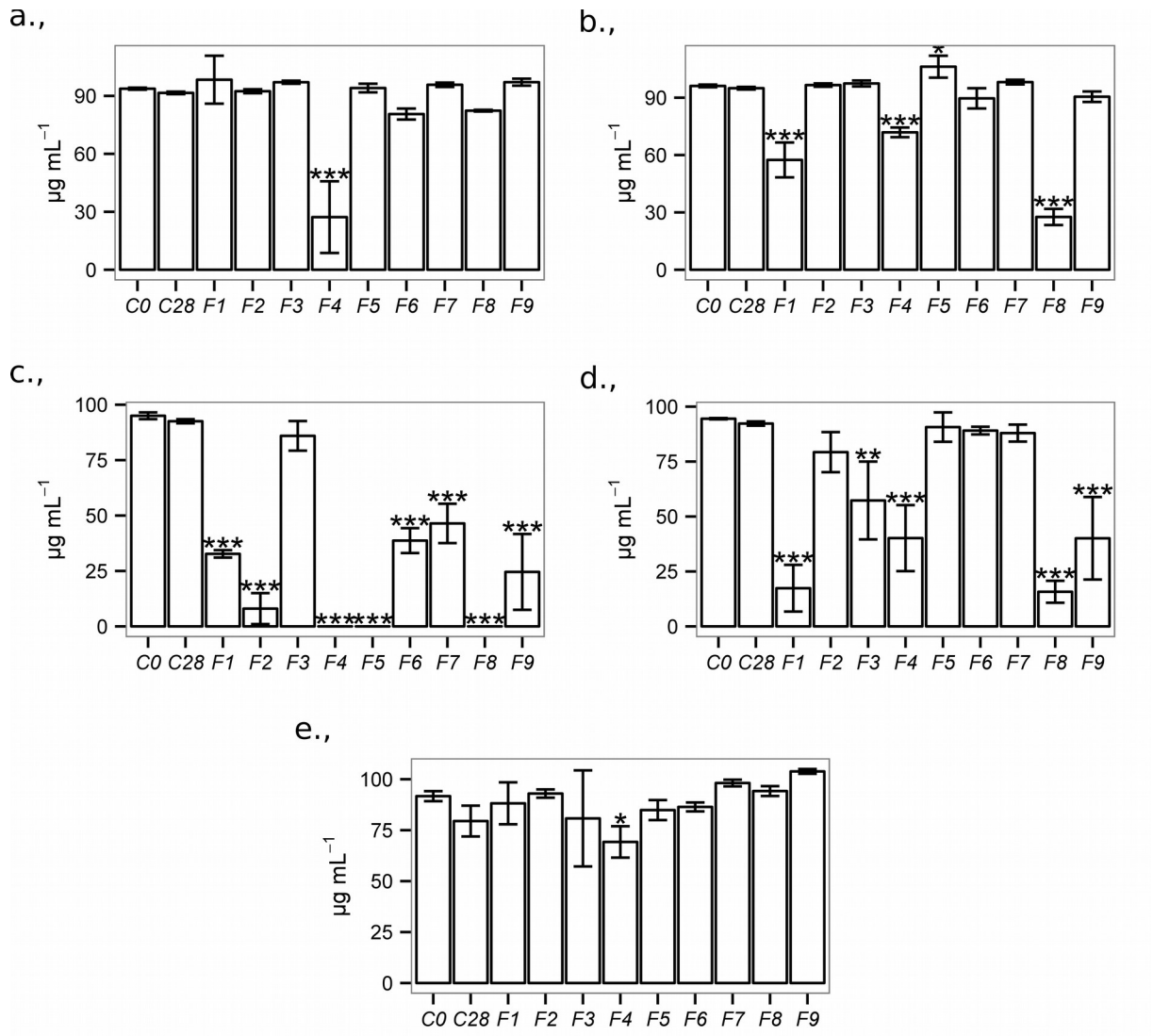
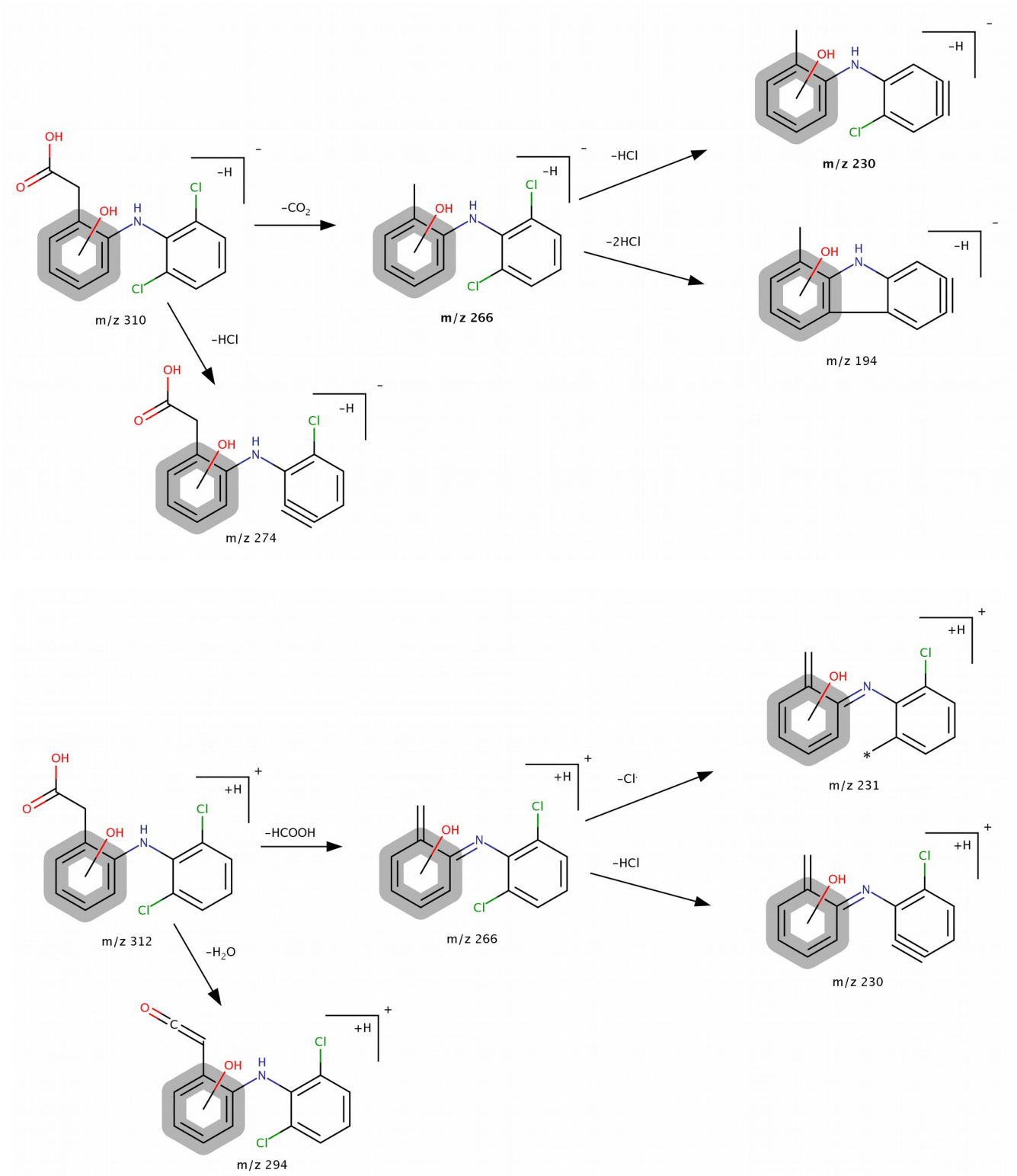


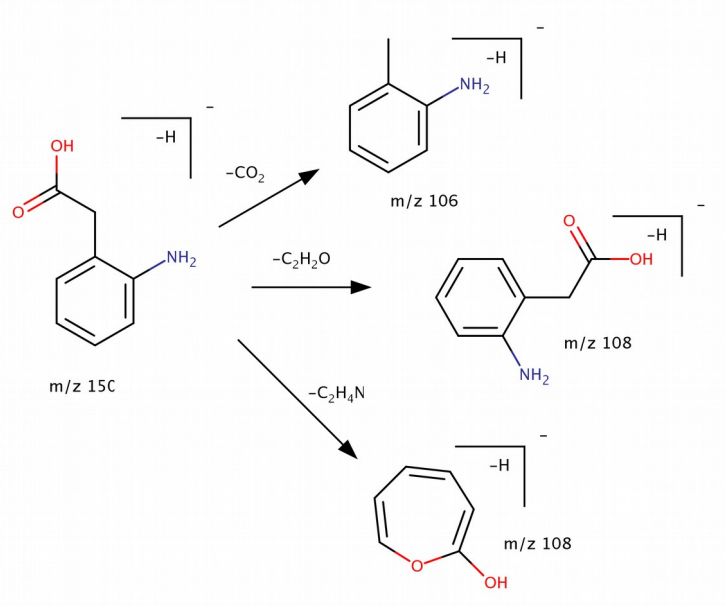
Fig.2. Residual NSAID concentrations in Czapek-Dox liquid media inoculated with plant-associated fungi, after 28 days of incubation. Initial concentration was 100 µg mL^{-1} for each NSAID. Subplots: a., diclofenac; b., diflunisal; c., ibuprofen; d., mefenamic acid; e., piroxicam. Abbreviations: C0, zero time; C28, end-time (28 days) control; F1-9, fungal strains No. ***1-9***. (28 days). Fungal strain codes: ***1***: *Aspergillus niger*; ***2***: *Eurotium repens*; ***3***: *Leptosphaerulina chartatum*; ***4***: *Aspergillus nidulans*; ***5***: *Eurotium amstelodami*; ***6***: *Cladosporium pseudocladosporioides*; ***7***: *Penicillium chrysogenum*; ***8***: *Bipolaris tetramera*; ***9***: *Epicoccum nigrum*. Statistical test: ANOVA, followed by post-hoc Dunnett's test, after ANOVA *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

Table 1. NSAID decomposition products formed by plant-associated fungi in model solutions, putatively identified by LC-ESI-MS. Only those peaks were shown, that could be successfully fragmented in MS^2. The first peak of the peak list in the MS^2 columns was subjected to further fragmentation to yield the MS^3 peaks. Abbreviations: DIC, diclofenac; DIF, diflunisal; IBU, iibuprofen; MEF, mefenamic acid; PIR, piroxicam. Fungal strains: ***1****, Aspergillus niger;* ***4****, Aspergillus nidulans;* ***8****, Bipolaris tetramera;* ***9****, Epicoccum nigrum*. Fragmentation pathway with structures can be found in the **Supplementary material**.

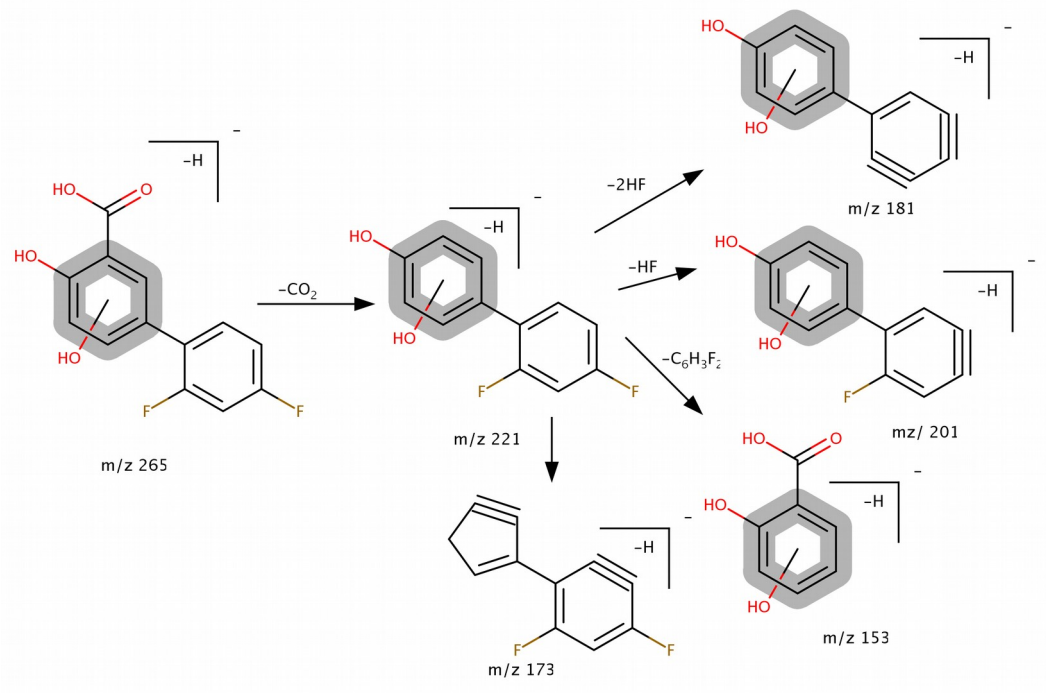
NSAID	$[M-H]^-$	MS^2 (-)	MS^3 (-)	$[M+H]^+$	MS^2 (+)	MS^3 (+)	Retention time (min.)	Putative identification	Fungal strain	References
DIC	310	266, 230, 274, 194	230, 194	312	294, 266, 230, 231	266, 230, 231	8.62	OH-DIC 1	***4, 8***	(Kallio et al., 2010), (Webster et al., 1998), (Madsen et al., 2008)
DIC	310	266, 274	230, 194, 215	n.d.			9.15	OH-DIC 2	***4, 8***	(Kallio et al., 2010)
DIC	150	106, 108	n.d.	n.d.			4.32	2-amino-phenylacetic acid	***4***	
DIF	265	221	173, 201, 153, 181	n.d.			7.69	OH-DIF	***4***	(Dickinson et al., 1994), (Macdonald et al., 1991)
IBU	221	177, 203	161	n.d.			6.41	OH-IBU	***1, 4, 8***	(Paíga et al., 2015)
MEF	256	212, 182	182, 194, 168, 92, 120, 106	258	240, 210, 222	222, 196, 240	8.18	OH-MEF 1	***4, 8, 9***	(Wing Lam et al., 2007)
MEF	256	212, 182	212, 106, 134	258	240, 210, 222	225, 212, 240, 222	8.55	OH-MEF 2	***4, 9***	(Wing Lam et al., 2007
PIR	346	282, 210, 178, 176	135, 109, 146, 264	348	111, 137, 180	111, 83	7.13	OH-PIR	***4***	(McKinney et al., 2004), (Tevell Åberg et al., 2009)
PIR	362	252, 226, 298, 162	224	364	111, 137, 180	n.d.	7.5	OH_2-PIR 1	***4***	(Tevell Åberg et al., 2009)
PIR	362	226, 162, 298, 183, 242, 135	208, 162	364	111, 137, 180, 210, 228	n.d.	8.57	OH_2-PIR 2	***4***	(Tevell Åberg et al., 2009)



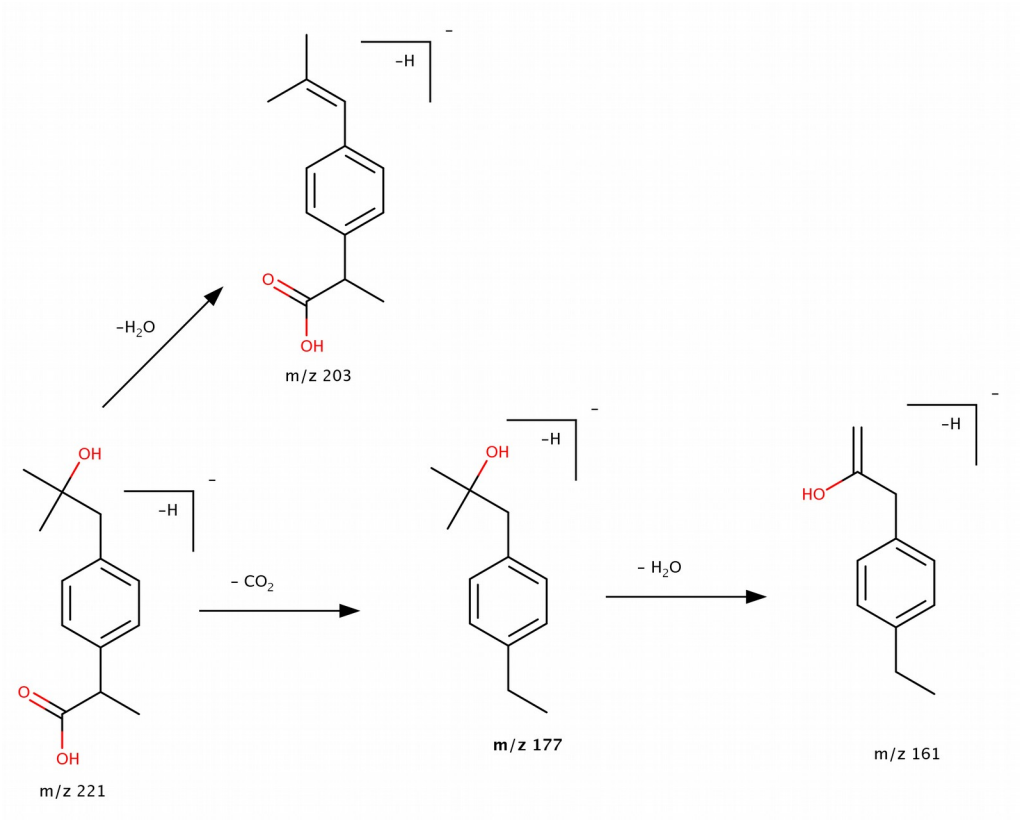
Supplementary Figure 1. Proposed fragmentation pathways of OH-diclofenac in negative ion mode, and positive ion mode.



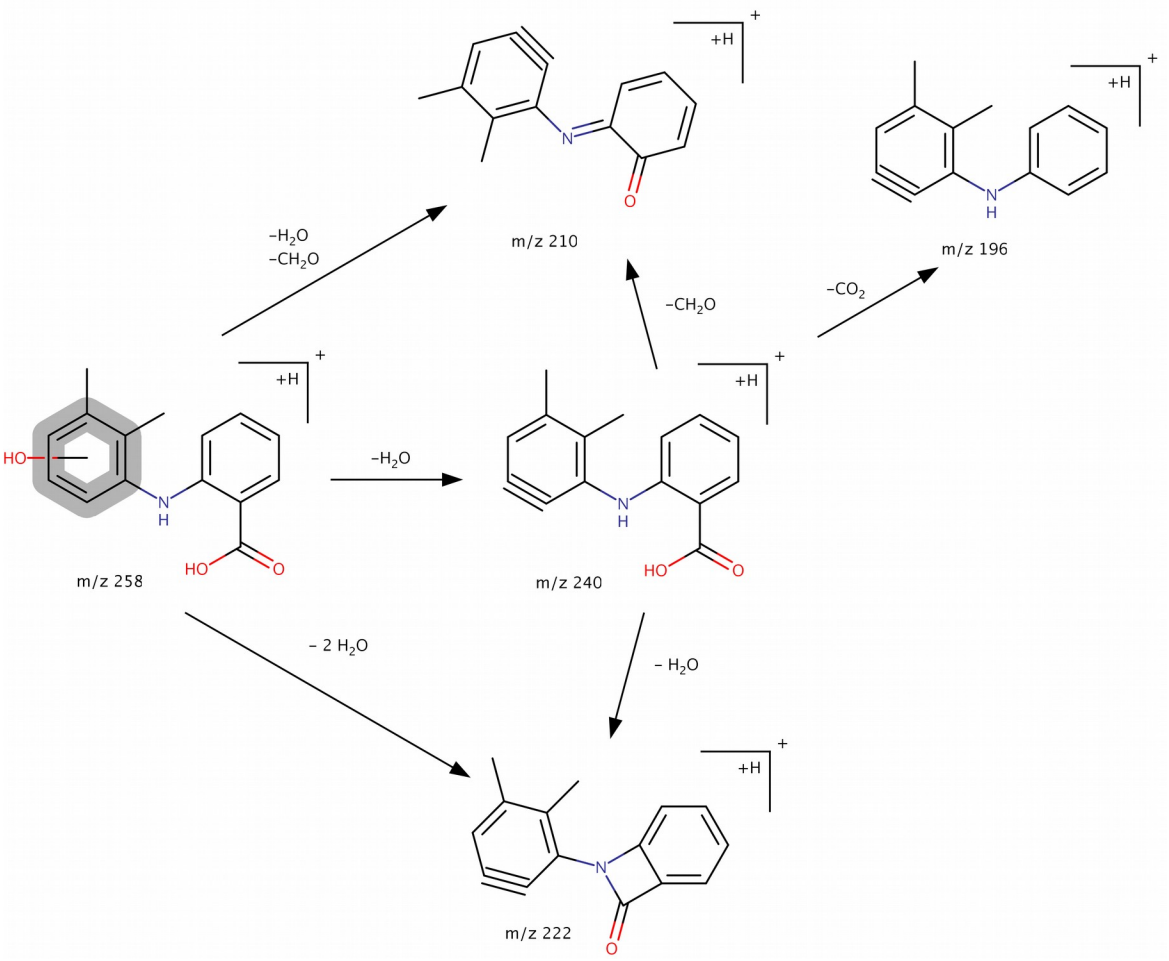
Supplementary Figure 2. Proposed fragmentation pathways of 2-aminophenylacetic acid in negative ion mode.



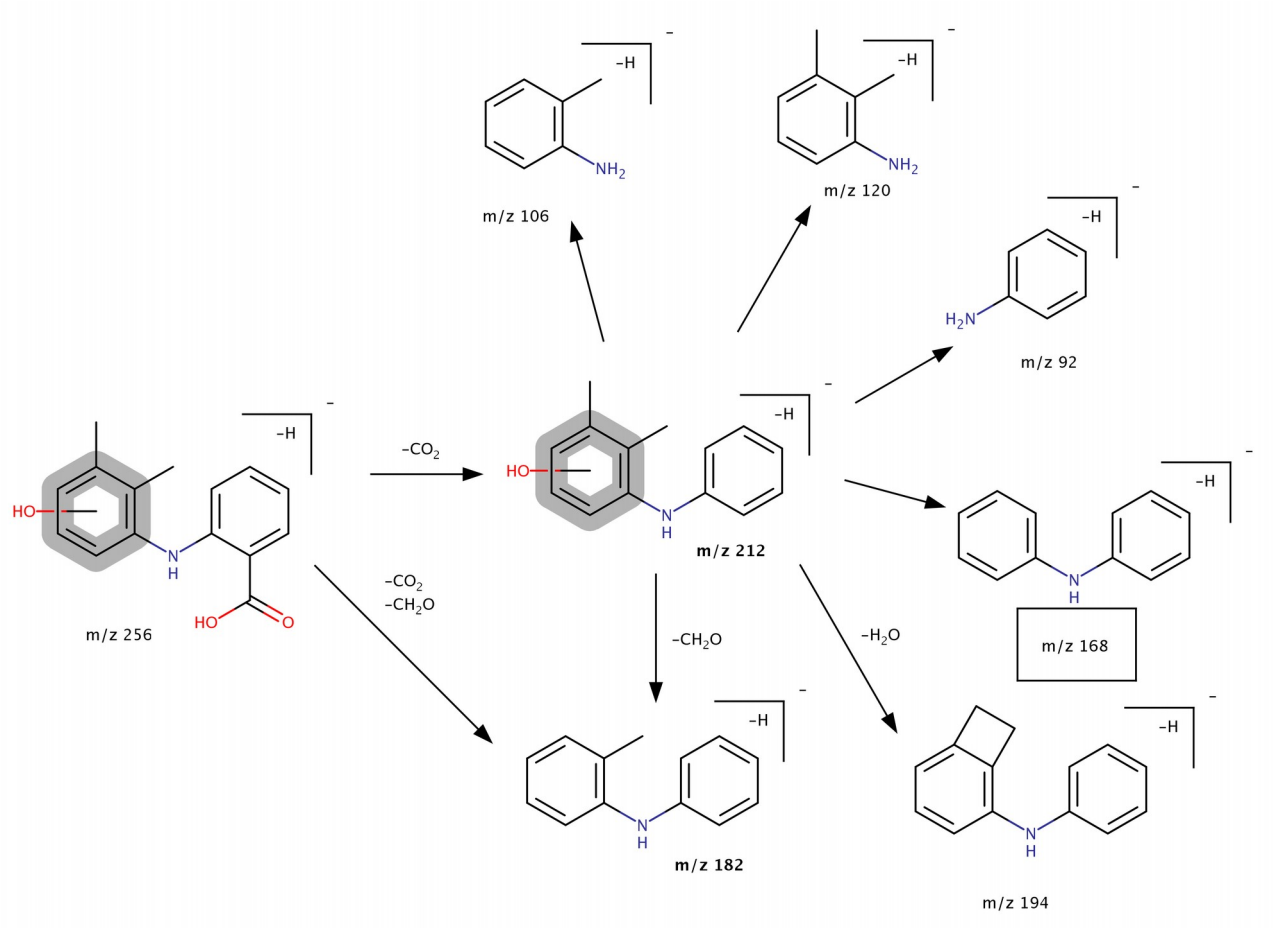
Supplementary Figure 3. Proposed fragmentation pathways of OH-diflunisal in negative ion mode.



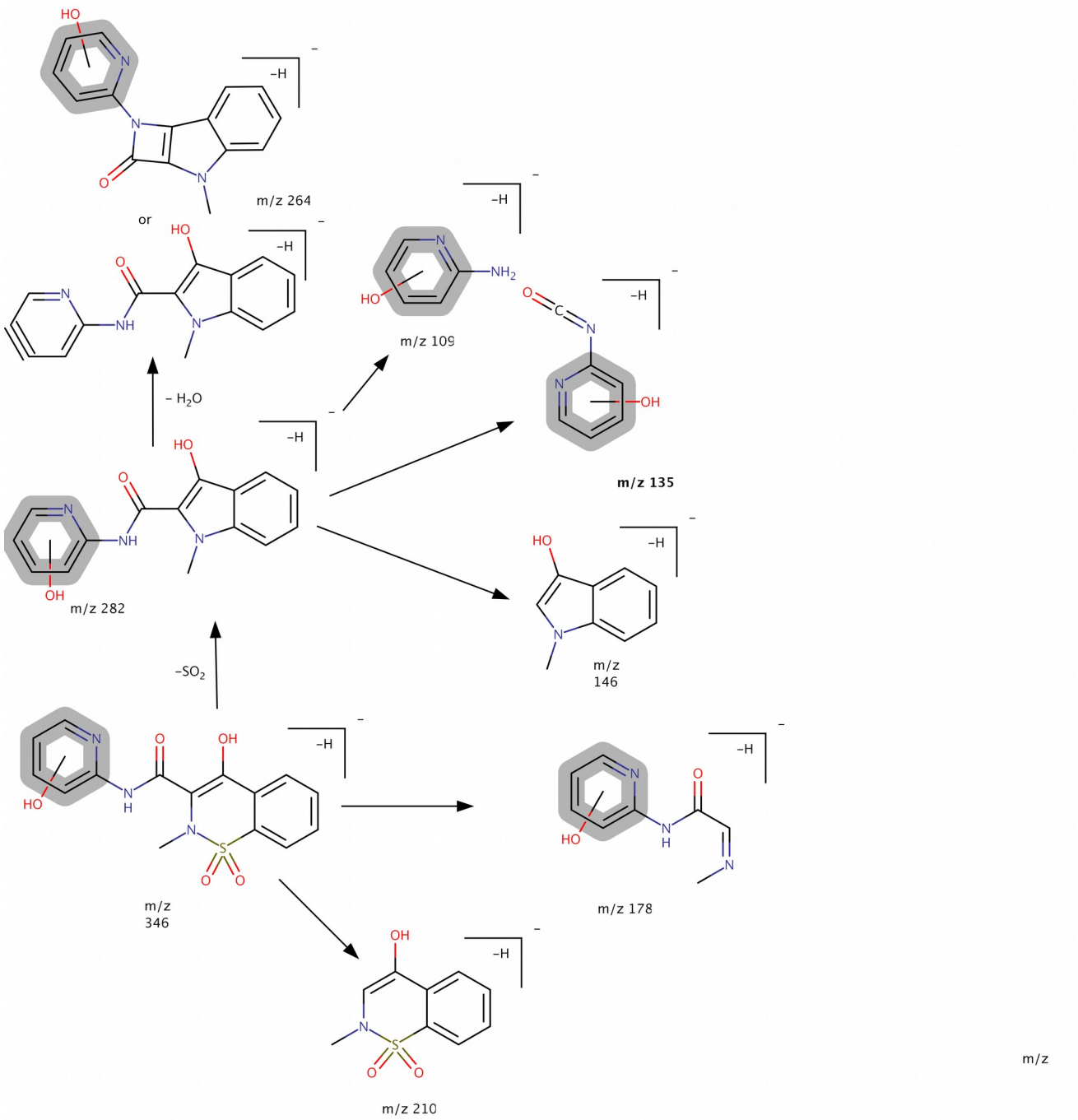
Supplementary Figure 4. Proposed fragmentation pathways of OH-ibuprofen in negative ion mode.



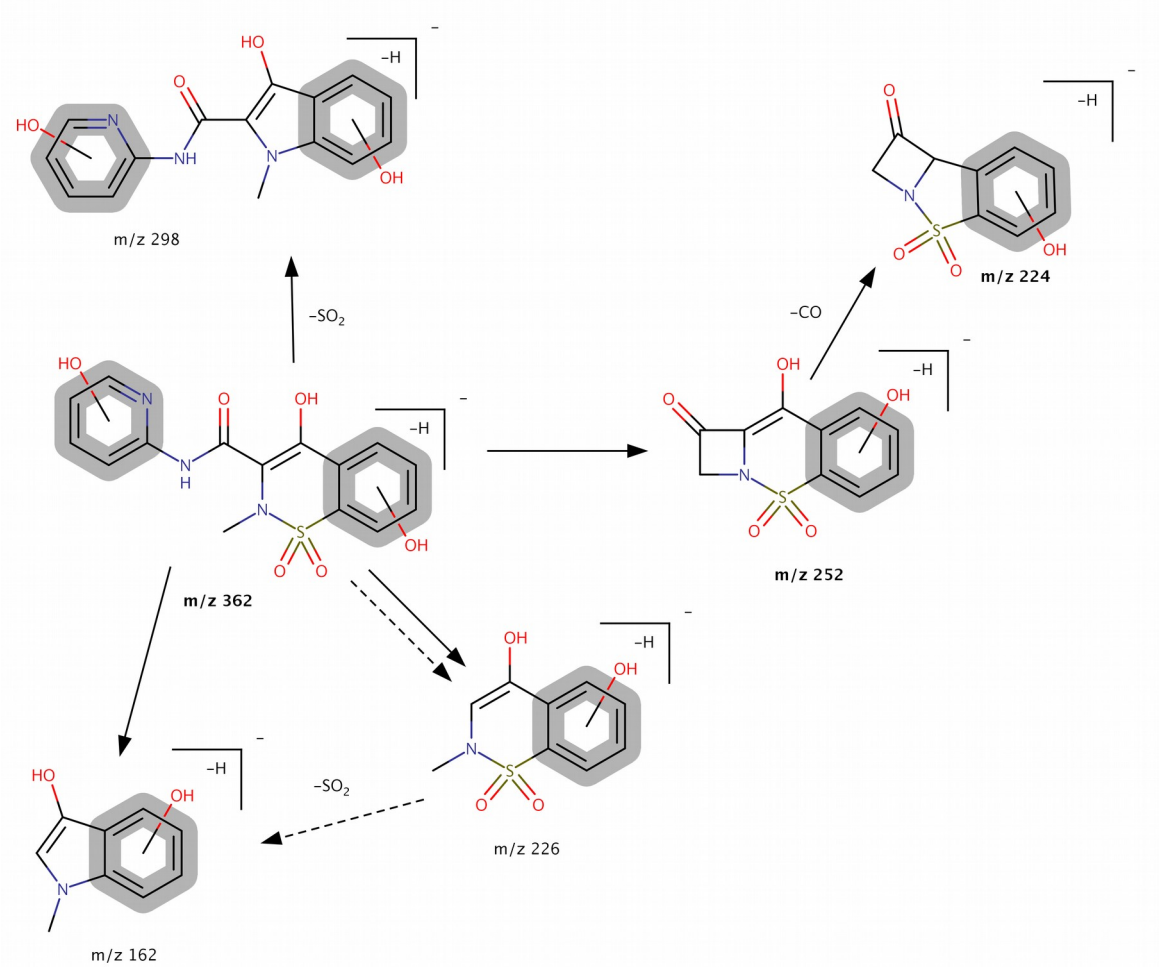
Supplementary Figure 5.a. Proposed fragmentation pathways of OH-mefenamic acid in positive ion mode.



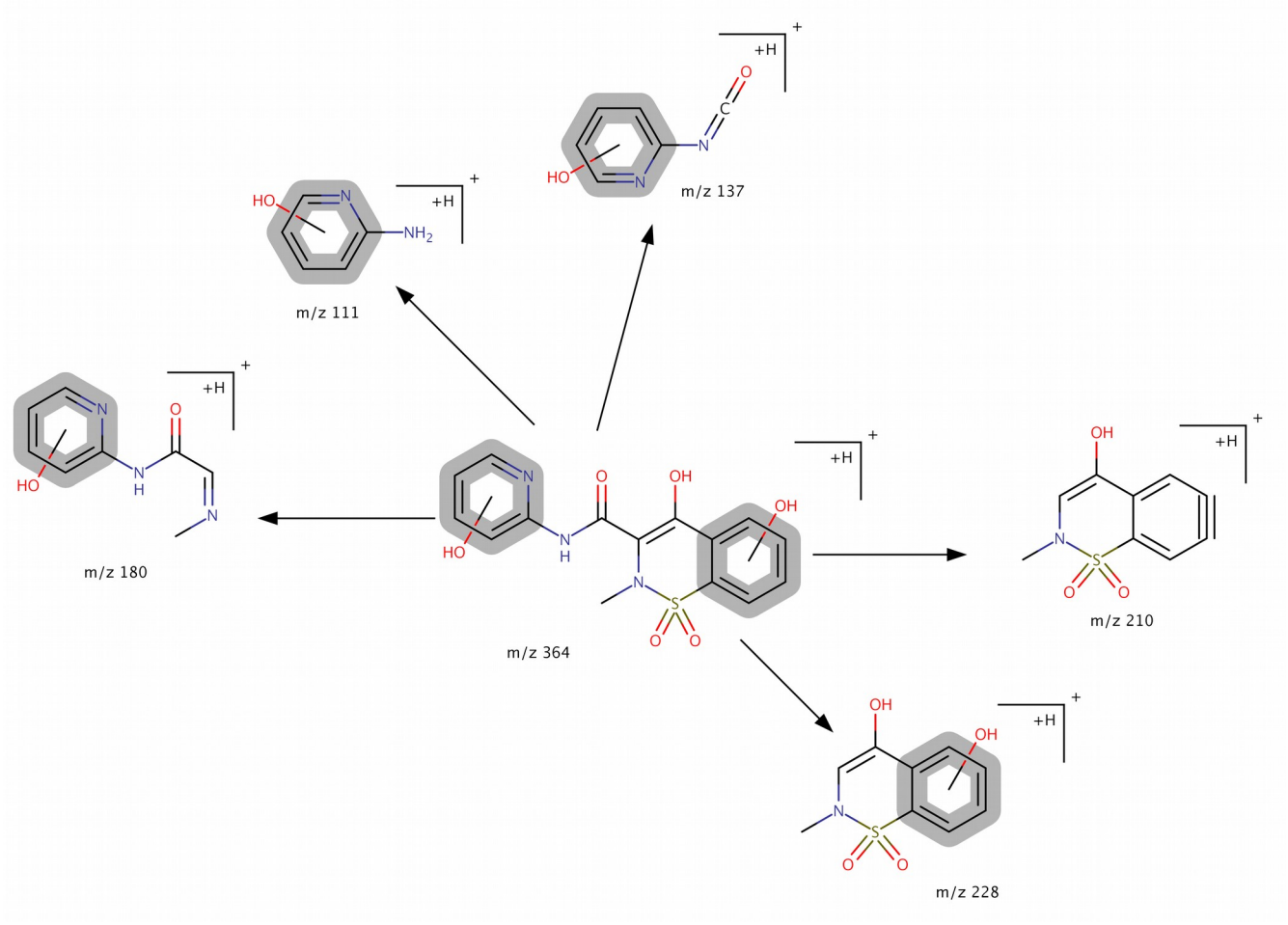
Supplementary Figure 5.b. Proposed fragmentation pathways of OH-mefenamic acid in negative ion mode.



Supplementary Figure 6. Proposed fragmentation pathways of OH-piroxicam in negative ion mode.



Supplementary Figure 7.a. Proposed fragmentation pathways of OH_2-piroxicam in negative ion mode.



Supplementary Figure 7.b. Proposed fragmentation pathways of OH_2-piroxicam in positive ion mode.