Supporting information

Metabolite Identification of a Radiotracer by Electrochemistry Coupled to Liquid Chromatography with Mass Spectrometric and Radioactivity Detection

*Anne Baumann[1], Andreas Faust[2,3], Marylin P. Law[2], Michael T. Kuhlmann[2], Klaus Kopka[3], Michael Schäfers[2], Uwe Karst[1]**

[1]University of Münster, Institute of Inorganic and Analytical Chemistry,

Corrensstraße 30, 48149 Münster

[2]University of Münster, European Institute for Molecular Imaging - EIMI,

Mendelstraße 11, 48149 Münster

[3]University Hospital of Münster, Department of Nuclear Medicine,

Albert-Schweitzer-Str. 33, 48149 Münster

ABSTRACT (Supporting information)

Based on the oxidation in an electrochemical cell, potential oxidative metabolites of the radiotracer (isatin) are generated. Using LC/ESI-ToF-MS, exact masses and molecular formulas of the metabolites are calculated, and for structure elucidation, LC/ESI-ion trap MS^n is utilized. Supplementary to the experimental section in the article, tables S-1 and S-2 provide the MS conditions applied for ESI-ToF-MS and ESI-ion trap MS^n experiments. Moreover, additional information about the RLM incubation mixture and the sample preparation of the body fluids from a mouse are given. As supporting information to the 'results and discussion' section, the fragmentation patterns of the isatin and the oxidation products *m/z* 507 (isatin+2O) and 477 (isatin+2H) are shown and discussed in following (figure S-1 to S-3). Moreover, the fragment ions used for structure elucidation of the further oxidation products (*m/z* 491, 493, 323, 339, 355, 463) are summarized in figure S-4.

EXPERIMENTAL (Supporting information)

MS conditions used for EC/ESI-ToF-MS and EC/LC/ESI-ToF-MS studies

Table S-1: ESI-ToF-MS parameters applied for the generation of mass voltammograms.

End plate offset [V]	-500	Skimmer 2 [V]	-24.6
Capillary [V]	3857	Hexapole 1 [V]	-26.7
Nebulizer gas (N_2) [bar]	0.8	Hexapole 2 [V]	-20.6
Dry gas (N_2) [L/min]	3	Hexapole RF [V]	100
Dry temperature [°C]	180	Transfer time [µs]	49
Capillary exit [V]	-135	Prepulse storage [µs]	1.2
Skimmer 1 [V]	-45	Detector [V]	-1300

For the EC/LC/ESI-ToF-MS analysis, the same parameters as provided in table S-1 were applied, except for the gases: Nebulizer gas: 0.9 bar, dry gas: 7 L/min. Calibration was performed using sodium formate cluster ions. For controlling the micrOTOF and data processing, micrOTOF control 1.1 and dataAnalysis 3 (Bruker Daltonics) software were used.

MS conditions used for EC/LC/ESI-ion trap MS^n experiments

For fragmentation experiments, the EC/LC system was connected to the ESI-ion trap MS^n Esquire 6000 (Bruker Daltonics). MS detection was carried out in the negative ion mode (table S-2).

Table S-2: ESI-ion trap MS^n parameters.

End plate offset [V]	-500	Skimmer [V]	-40
Capillary [V]	4000	Capillary exit [V]	-124.8
Nebulizer gas (N_2) [psi]	50	Lens 2 [V]	60
Dry gas (N_2) [L/min]	10	Octopole RF amplitude [V]	179.2
Dry temperature [°C]	300	Trap drive [%]	61.2
Oct 2 DC [V]	-1.7	Detector [V]	-1300
Oct 1 DC [V]	-12	ICC target	10000
Lens 1 [V]	5	Max. accu time [ms]	200
Skimmer [V]	-40	Scan *m/z*	100-600
Capillary exit [V]	-124.8	Fragmentation amplitude [V]	1

For controlling the LC/ESI-ion trap MS^n and data processing, micrOTOF control 6.2, Hystar 3.2 and dataAnalysis 4.0 (Bruker Daltonics) software were used.

Sample preparation

RLM incubation: Pooled male RLM (Sprague Dawley) with a protein concentration of 20 mg/mL and a total P450 enzyme activity of 520 pmol/mg were delivered by BD Bioscience (Woburn, MA, USA). A mixture of microsomal protein and the isatin, dissolved in 50 mM phosphate buffer solution (adjusted to pH 7.4), was preincubated for 5 min at 37 °C. Magnesium chloride and NADPH were added to the incubation mixture, which was then further incubated at 37 °C for 90 min. The total volume of the incubation mixture was 500 µL and the final concentrations were as follows: 1.3 mg/mL microsomal protein, 50 µM substrate added in DMSO, 0.5 mM magnesium chloride, 1.2 mM NADPH. Subsequent to the incubation, proteins were precipitated by adding an equal amount of ACN to the incubation mixture. After centrifugation (1701 x g), the supernatant was analyzed by LC/ESI-ToF-MS. Control incubations were carried out without adding NADPH.

In vivo studies with the non-radioactive isatin: In case of the urine sample, 5 µL were diluted with 75 µL 0.1% (v/v) acetic acid. Protein precipitation was carried out by adding 50 µL ACN to 50 µL serum and 15 µL ACN to 5 µL bile. After centrifugation of each sample (1701 x g) the supernatant was analysed, whereby the supernatant of the bile sample was diluted with 85 µL 0.1% (v/v) acetic acid prior to the analysis. The studies were carried out twice and comparative measurements were performed with body fluids from a mouse without administration of the isatin.

In vivo studies with the radioactive isatin: In case of bile, protein precipitation was performed as described above. The further samples (ACN liver tissue extract, urine, aqueous wash from the duodenum) were used without further dilution. After centrifugation of each sample the supernatant was analysed.

RESULTS AND DISCUSSION (Supporting information)

Fragmentation of the isatin

Aiming at the structure elucidation of the isatin and its oxidation products, fragmentation experiments (MS^2 and MS^3) were carried out. The MS^2 fragmentation pattern of the isatin and the MS^3 pattern of the fragment *m/z* 449 are presented in figure S-1.

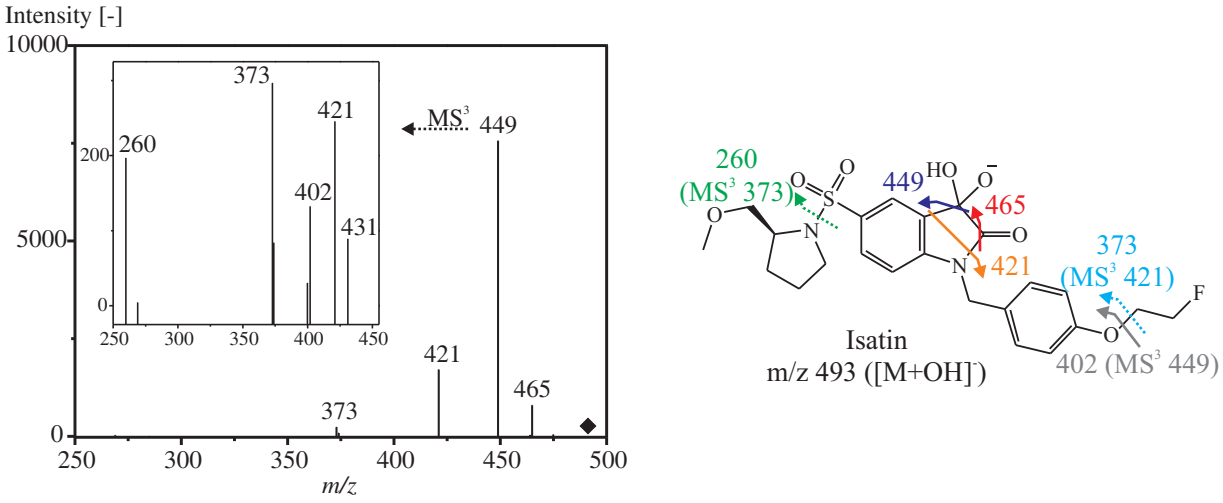

Figure S-1: Fragmentation of the isatin (EC/LC/ESI-ion trap MS^n). Fragment ions *m/z* 465, 449, 421 also occurred upon in-source fragmentation in ESI-ToF-MS measurements. The determined molecular formulas are in agreement with the calculated molecular formulas of the fragments indicated above (deviation < 5ppm).

The fragmentation results in the loss of CO (*m/z* 465), CO_2 (*m/z* 449) and C_2O_3 (*m/z* 421) from the precursor ion (*m/z* 493, $[M+OH]^-$). The loss of CO proves that the isatin is detected in the hydrated form and not as isatinate. Due to the carboxylic acid group of the isatinate, a fragmentation would result in the loss of CO_2 and C_2O_3 but not CO. The fragmentation reaction which leads to *m/z* 449 is assumed to proceed via a proton and charge transfer from the hydroxyl group to the aromatic ring and thus the neutral loss of CO_2. The MS^3 of *m/z* 449 pattern reveals that *m/z* 421 is formed via the loss of CO from

m/z 449. Further fragmentation reactions take place at the nitrogen-sulfur bond (*m/z* 260) and at the phenolic oxygen atom (*m/z* 373, 402).

Fragmentation of the metabolite m/z 477

Figure S-2 shows the fragmentation pattern of the oxidation product *m/z* 477, obtained by EC/LC/ESI-ion trap MS^n.

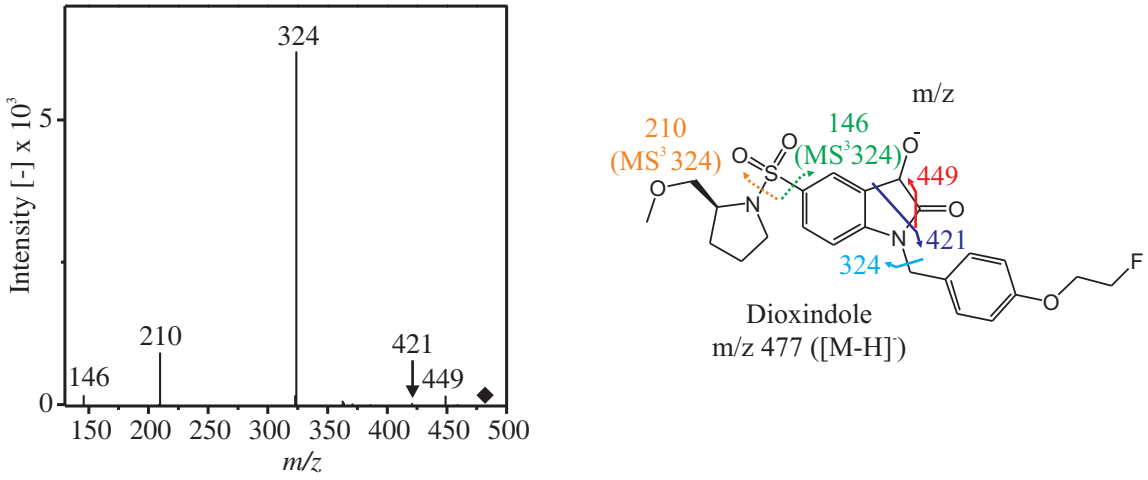

Figure S-2: Fragmentation of the oxidation product *m/z* 477 (EC/LC/ESI-ion trap MS^n).

The fragment ions *m/z* 324, 449 and 210 also occurred upon in-source fragmentation in EC/LC/ESI-ToF-MS measurements. Based on these data, the molecular formulas were calculated and are in agreement with the fragments indicated in figure S-2 (deviation between the measured and calculated molecular formulas < 5ppm). The detected fragments are in agreement with the postulated dioxindole structure, shown below the spectrum. The fragments *m/z* 146 and 324 prove that the modification (+2H) has taken place in the isatin moiety. The loss of CO and not CO_2 (*m/z* 449) shows that no carboxyl group (isatinate) has been formed. In contrast to the fragmentation of the isatin itself (figure S-1), not the loss of C_2O_3 but the loss of C_2O_2 (*m/z* 421) is found, which again supports the dioxindole structure. As discussed in the article, it is assumed that the isatin is transformed into the dioxindole via a hydroxyl radical driven mechanism.

Fragmentation of the metabolite m/z 507

Figure S-3 shows the fragmentation of the oxidation product *m/z* 507, obtained by EC/LC/ESI-ion trap MS^n. The fragment ions *m/z* 479, 463, 435, 257 also occurred upon in-source fragmentation in EC/LC/ESI-ToF/MS analysis, and the determined molecular formulas are in agreement with the formulas of the fragments indicated in figure S-3 (deviation < 5ppm).

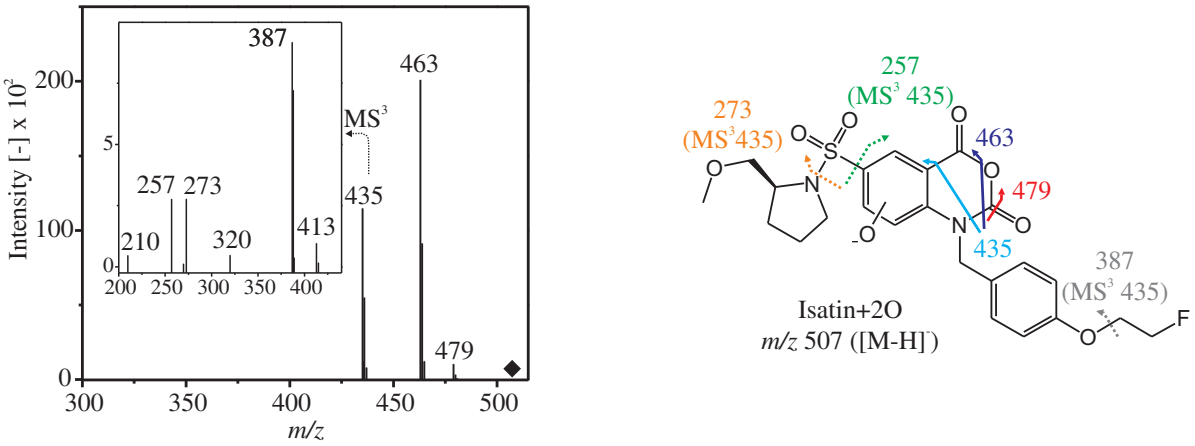

Figure S-3: Fragmentation of the oxidation product *m/z* 507 (EC/LC/ESI-ion trap MS^n).

The comparison of the MS^2 fragmentation of the $[M+OH]^-$ ion of the isatin (figure S-1) with the fragmentation pattern of *m/z* 507 reveals characteristic similarities. Both compounds fragment via a neutral loss of CO, CO_2 and C_2O_3, which indicates in both cases the presence of a third oxygen atom in the isatin moiety. In case of the isatin, an additional OH group at the C-3 atom has been postulated. In terms of the oxidation product *m/z* 507, the molecular formula unveils solely the gain of oxygen but not hydrogen. Hence, the presence of a hydrated isatin species, as discussed in figure S-1, can be excluded. In contrast, the fragmentation pattern of *m/z* 507 is in consistence with the formation of an isatoic anhydride. In terms of the second oxygen atom, the fragment ions *m/z* 257, 435 and 387 limit its possible position in the molecule. However, no exact position can be given. Due to the fact that a two times oxygenated species of the *N*-dealkylation product *m/z* 323 occurs as well (*m/z* 355, figure 5), it is likely that the oxygenation does not take place in the *N*-alkyl residue. Thus, it is suggested that a hydroxylation in the aromatic ring of the isatin moiety has taken place.

Fragmentation of the further metabolites

The fragmentation and the derived structures of the oxidation products *m/z* 323, 339, 355, 493 and 491 are summarized in figure S-4.

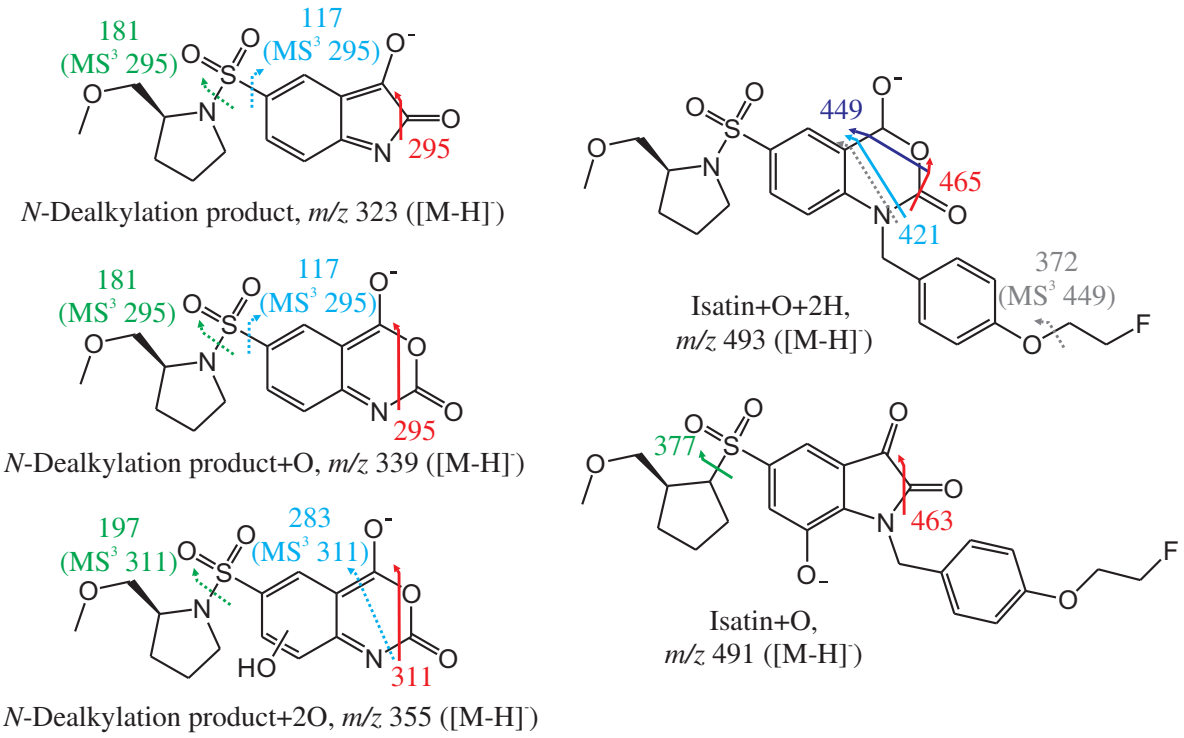

Figure S-4: Structures derived from the fragmentation of the products *m/z* 323, 339, 355, 493 and 491. In case of *m/z* 323, the isomer eluting at 5.3 min was studied (figure 4).

The fragmentation of the *N*-dealkylation products *m/z* 323, 339 and 355 correlates with the formation of an isatoic anhydride for *m/z* 339 and 355. In case of *m/z* 355, the MS^3 fragment ions *m/z* 283 and *m/z* 197 prove that the second oxygen atom is located in the aromatic ring of the isatin. The fragmentation pattern and the structure of *m/z* 355 are in good accordance with the discussed oxidation product *m/z* 507. The oxidation product *with m/z* 493 has the same molecular formula and the same MS^2 fragmentation pattern as the $[M+OH]^-$ ion of the isatin (S-1). This finding indicates that, again, a hydrated isatin is present, in this case with an OH group at the C-2 atom and not the C-3 atom. However, from the literature it is known that a hydroxylation at C-2 results in a ring opened isatinate structure[28]. Upon fragmentation, the isatinate carboxyl group would not result in the observed loss of CO, but solely

in the loss of CO_2 and C_2O_3. Hence, instead of a hydrated or isatinate structure, it is postulated that an isatoic anhydride derivative has been formed. As demonstrated in figure S-4, the fragments are in good agreement with the proposed structure.

Finally, the fragmentation of the oxidation product *m/z* 491 (isatin+O) has been studied. Because of low product ion intensities, only two fragment ions could be observed, which both correlate with the structure shown in figure S-4. In particular, the absence of the neutral loss of CO_2 and C_2O_3 supports the assumption that in this case, the additional oxygen atom is located in the aromatic part of the isatin and not between the two carbonyl functions of the isatin. Furthermore, the formation of an isatoic anhydride can be excluded, since it would not be detectable as $[M-H]^-$ ion in the ESI negative ion mode. In contrast to the postulated isatoic anhydrides *m/z* 493 and 507, an isatoic anhydride of *m/z* 491 would not provide a site for deprotonation and hence formation of the $[M-H]^-$ ion.