Electronic Supplementary Material (ESI) for Analytical Methods

Supplementary Information

Using Electrochemistry for Metabolite Simulation and Synthesis in Preventive Doping Research: Application to the Rycal S107 and the PPARδ-agonist GW1516

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Abstract (Supplementary Information)

Biotransformation of the two potential doping agents S107 and GW1516 was simulated in an electrochemical (EC) cell and compared to results from *in vitro* experiments. Supplementary to the article, details on experimental conditions, instrumental setups and product ion spectra are given (or discussed) in the following.

Experimental (Supplementary Information)

In support of the "Experimental" section in the article, Tables S-1 through S-9 provide the different LC separation and ESI-MS conditions used during the studies with (D_3-)S107 and GW1516.

LC Separation Conditions

Studies with (D_3-)S107

Table S-1: LC gradient profile applied for all (EC/)LC/ESI-MS analyses of S107 and its stable isotope-labeled analog D_3-S107 (in combination with column 1 and solvent composition 1)*.

Time [min]	0	10	11	12	12.01	16	16.01	18
Acetonitrile [%]	2	70	100	100	2	2	2	2
Flow Rate [µL]	300	300	300	300	400	400	300	300

Studies with GW1516

Table S-2: LC gradient profile employed for (EC/)LC/ESI-QqLIT-MS and (EC/)LC/ESI-Orbitrap-MS analyses of GW1516 (in combination with column 1 and solvent composition 1)*.

Time [min]	0	8	10	12	12.01	18
Acetonitrile [%]	30	80	100	100	30	30
Flow Rate [µL]	300	300	300	300	300	300

Table S-3: LC gradient profile used for (EC/)LC/ESI-QTOF-MS analyses of GW1516 (in combination with column 2 and solvent composition 2)**.

Time [min]	0	8	10	12	12.01	18
Acetonitrile [%]	30	80	100	100	30	30
Flow Rate [µL]	300	300	300	300	300	300

General

During all LC separations conducted in this work, the injection volume was 5 µL and the column oven temperature was set to 35 °C.

*Column 1: Phenomenex Kinetex RP-C_{18} analytical column, 100 mm length x 2.1 mm i. d., 2.6 µm particle size (Aschaffenburg, Germany)

**Column 2: Thermo Scientific Hypersil Gold column, 50 mm length × 2.1 mm i. d., 1.9 µm particle size (Bremen, Germany)

*Solvent Composition 1:	A	5 mM NH_4OAc buffer (adjusted to pH 3.5 with 0.1% HOAc)
	B	ACN
**Solvent Composition 2:	A	0.1% FA
	B	ACN

ESI-MS Conditions

micrOTOF – EC/ESI-TOF-MS Studies ("Mass Voltammograms", On-Line)

Table S-4: ESI-TOF-MS conditions applied for the generation of mass voltammograms of S107. Detection was carried out in positive ionization mode within a range of *m/z* 50 – 1000.

ESI-TOF-MS Parameters (S107)			
End Plate Offset [V]	– 400	Hexapole 1 [V]	25.7
Capillary [V]	– 4000	Hexapole 2 [V]	20.3
Nebulizer (N_2) [bar]	0.8	Hexapole RF [Vpp]	90
Dry Gas (N_2) [L/min]	6.0	Transfer Time	49
Dry Temperature [°C]	180	Pre Pulse Storage [µs]	1
Capillary Exit [V]	120	Lens 1 Storage [V]	30
Skimmer 1 [V]	40	Lens 1 Extraction [V]	20.6
Skimmer 2 [V]	23.6	Detector [V]	0

For GW1516, the same conditions were used, except for the parameters listed in the subsequent Table S-5.

Table S-5: Selected ESI-TOF-MS parameters (differing from those in Table S-4) for the generation of mass voltammograms of GW1516. Detection was carried out in positive ionization mode within a range of *m/z* 50 – 1000.

ESI-TOF-MS Parameters (GW1516)			
Capillary Exit [V]	90	Hexapole 1 [V]	26.7
Skimmer 1 [V]	30	Hexapole 2 [V]	20.6
Skimmer 2 [V]	24.6	Lens 1 Extraction [V]	20.9

Measurements of each mass voltammogram were recorded in triplicate for both substances to ensure reproducibility of the results. Internal calibration was accomplished using sodium formate clusters leading to mass accuracies < 5 ppm.

API 2000 QTRAP – (EC, *In Vitro*/)LC/ESI-QqLIT-MS Studies (On-, Off-Line)

Screening for metabolites was carried out using positive ionization and full scan mode (EMS mode) in a range of *m/z* 100 – 400 for S107 and D_3-S107 or of *m/z* 100 – 600 for GW1516. For fragmentation, enhanced product ion spectra (EPI mode) were recorded.

Table S-6: Summary of ESI(+)-QqLIT-MS conditions employed during this project. EMS: enhanced MS (full scan), EPI: enhanced product ion scan.

ESI-QqLIT-MS Parameters	EMS Mode		EPI Mode
	(D_3-)S107	*GW1516*	*all*
Ionization Spray Voltage [V]	4500	4500	4500
Source Temperature [°C]	350	350	350
Curtain Gas (N_2) [psi]	25	25	25
Nebulizer Gas (N_2) [psi]	60	60	60
Drying Gas [psi]	30	30	30
Entrance Potential (EP) [V]	10	10	10
Collision-Activated Dissociation (CAD) Gas	"High"	"High"	"High"
Declustering Potential (DP) [V]	70	20	20
Collision Energy (CE) [V]	10	5	25

Additionally, EMS and EPI experiments were performed in negative ionization mode for GW1516 while applying the same conditions as described above, albeit with reversed polarity. Nitrogen gas (5×10^{-3} Pa) was delivered by a nitrogen generator (CMC Instruments, Eschborn, Germany).

Exactive – EC/LC/ESI-Orbitrap-MS Studies (Off-Line)

For studies with GW1516, the Exactive was operated either in positive or in negative ionization mode and calibrated using the manufacturer's calibration mixture (yielding a total of seven reference masses). Mass accuracies better than 5 ppm in both ionization modes were achieved throughout the study. S107 and D_3-S107 were analyzed in positive ionization mode only. The same parameters were applied for all three substances as presented in Tables S-7 and S-8.

Table S-7: General ESI-Orbitrap-MS conditions applied for off-line analyses of (D_3-)S107 and GW1516 in this work.

Orbitrap-MS Parameters	ESI(+)	ESI(−)
Ionization Spray Voltage [V]	5500	− 4500
Capillary Temperature [°C]	275	275
Sheath Gas Flow Rate (N_2) [a.u.]	20	20
Aux Gas Flow Rate (N_2) [a.u.]	2	2
Capillary Voltage [V]	50	− 25
Tube Lens Voltage [V]	150	− 100
Skimmer Voltage [V]	20	− 20

Table S-8: Summary of different ESI-Orbitrap-MS settings employed during off-line analyses with (D_3-)S107 and GW1516. FWHM: full width at half maximum, HCD: higher-energy collision-induced dissociation.

Setting	*m/z* Range	Resolution [FWHM]	HCD Scan [V]
a)	100 – 1500	50'000	–
b)	90 – 1000	25'000	25
c)	70 – 550	25'000	50

Nitrogen gas (5 x 10^{-3} Pa) was delivered by a nitrogen generator (CMC Instruments, Eschborn, Germany).

TripleTOF – (EC, *In Vitro*)LC/ESI-QTOF-MS Studies (Off-Line)

A more detailed investigation of GW1516 samples on the QTOF mass spectrometer was carried out using the positive ionization mode only. Before sample injection, mass calibration was achieved by post-column T-split infusion of the AB Sciex APCI positive calibration solution using the external calibrant delivery system. Metabolite screening was conducted in full scan mode (range of *m*/*z* 100 – 1100), while further characterization was achieved via MS/MS experiments of selected precursor ions.

Table S-9: ESI(+)-QTOF-MS conditions used for studies with GW1516. EMS: enhanced MS (full scan), EPI: enhanced product ion scan.

ESI-QTOF-MS Parameters	EMS Mode	EPI Mode
Ionization Spray Voltage [V]	5500	5500
Source Temperature [°C]	500	500
Curtain Gas (N_2) [psi]	25	25
Nebulizer Gas (N_2) [psi]	50	50
Drying Gas [psi]	70	70
Declustering Potential (DP) [V]	80	80
Collision Energy (CE) [V]	10	35

Nitrogen gas (5 x 10^{-3} Pa) was delivered by a nitrogen generator (CMC Instruments, Eschborn, Germany).

Results and Discussion (Supplementary Information)

Supplementary to the "Results and Discussion" section in the article, instrumental setups used in this work (Figure S-1) and selected ESI-MS/MS spectra (Figures S-2 through S-5) are illustrated in the following for an improved understanding of the text. Product ion spectra will be presented for S107 (Figure S-2) and GW1516 (Figure S-3) as well as for their EC-generated oxidation products S1d, S1e, S2 and S4 (Figure S-4) or G2d and G2e (Figure S-5), respectively. Moreover, the structural discussion of the two mono-oxygenated metabolite isomers S1b and S1c is provided.

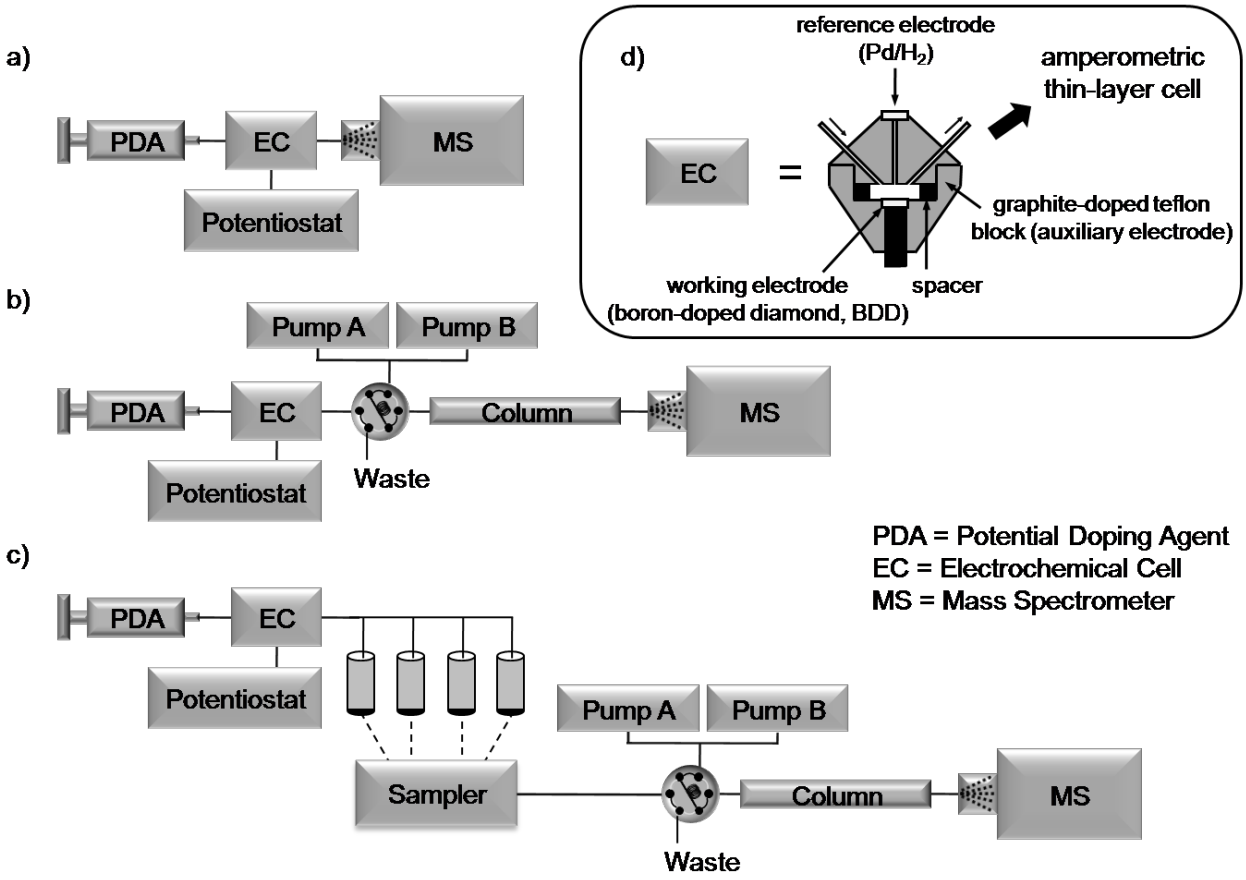

Figure S-1: The three different instrumental setups that were employed during this study are shown including the coupling of a) on-line EC/ESI-MS, b) on-line EC/LC/ESI-MS and c) off-line EC/LC/ESI-MS. Geometry and technical details of the amperometric thin-layer cell itself are schematically depicted in part d).

S107

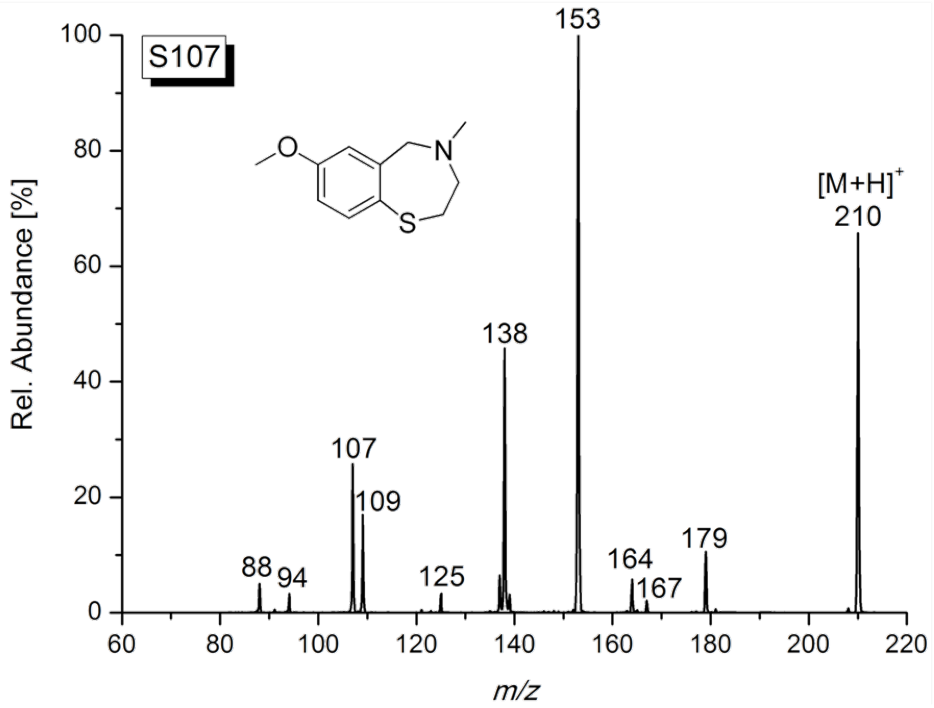

Figure S-2: ESI-MS/MS spectrum of the $[M+H]^+$ ion of "Rycal" S107 recorded on a QqLIT-MS instrument (QTRAP, Applied Biosystems, Darmstadt, Germany).

Figure S-2 is in accordance with data from Thevis et al. (cf. ESI product ion spectra in reference 8 [M. Thevis, S. Beuck, A. Thomas, M. Kohler, N. Schlörer, I. Vajiala and W. Schänzer, *Drug Test. Anal.*, 2009, **1**, 32-42] and reference 10 [M. Thevis, S. Beuck, A. Thomas, B. Kortner, M. Kohler, G. Rodchenkov, W. Schänzer, *Rapid Commun. Mass Spectrom.* 2009, **23**, 1139-1146]).

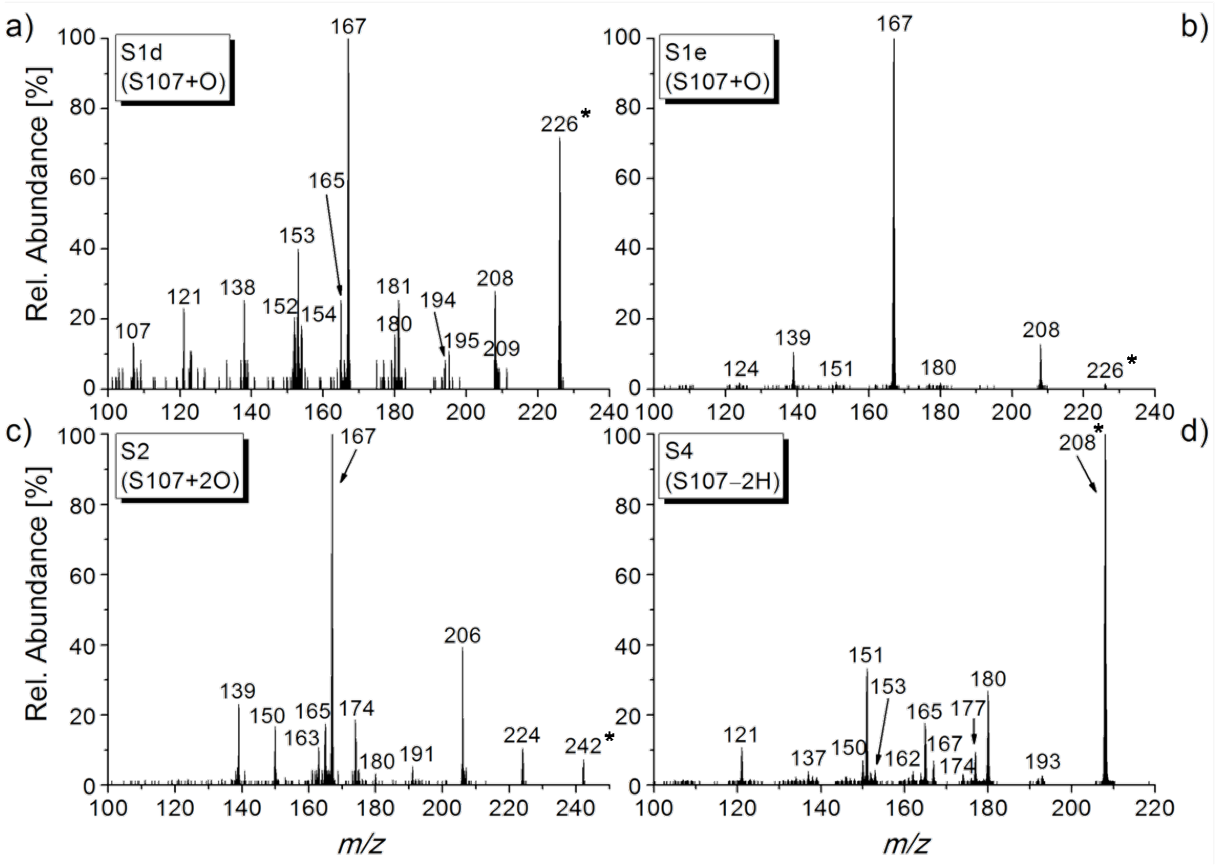

Figure S-3: EPI mass spectra of selected, EC-generated metabolites of S107 (acquired via LC/ESI-MS/MS analysis on an API 2000 QTRAP mass spectrometer in the positive ionization mode): a) mono-oxygenated isomer S1d (*N*-oxide), b) mono-oxygenated isomer S1e (benzylic hydroxylation), c) bis-oxygenated product S2, d) dehydrogenated product S4. $[M+H]^+$ ions of the parent compounds are marked with an asterisk.

Structural discussion of mono-oxygenated metabolite isomers S1b and S1c

In the MS^2 spectrum of S1b (cf. Table 3 in the article, data not shown), a main product ion was detected at *m/z* 169 which possibly corresponds to an oxygenated type of the characteristic S107 fragment at *m/z* 153. As the latter consists of a 3-methoxytoluene moiety still carrying the sulfur atom[8], the presence of a metabolite that was hydroxylated at the aromatic ring of the 1,4-benzothiazepine-based structure, is supported. Besides, *m/z* 153 was no longer detected. For S1c, however, collision-induced dissociation (CID) led to the typical fragments *m/z* 153 and *m/z* 138, hence, rendering aromatic hydroxylation at another C-atom unlikely. Next to the loss of water (*m/z* 208), major signals were found at *m/z* 164, *m/z* 166, and *m/z* 180 in this case. Since these ions all have an even electron configuration, the nitrogen atom should still be present in the molecule according to the nitrogen rule (considering non-radical species). MS^2 experiments with the corresponding oxygenated D_3-S107 isomer unveil the generation of product ions at *m/z* 167, *m/z* 169 and *m/z* 183, indicating retention of the deuterated *N*-methyl group as well. Thus, it is proposed that these species are produced as a result of rearrangement processes in the seven-membered thiazepine ring, following the formal loss of CH_2SO (62 u, *m/z* 164), C_3H_8O (60 u, *m/z* 166), and C_2H_6O (46 u, *m/z* 180), respectively. This leads to the conclusion of S1c being most probably hydroxylated at one of the two saturated carbon atoms which are located between the sulfur (*S*-1) and the nitrogen (*N*-4) position of the 1,4-benzothiazepine framework (cf. Figure 4 in the article).

GW1516

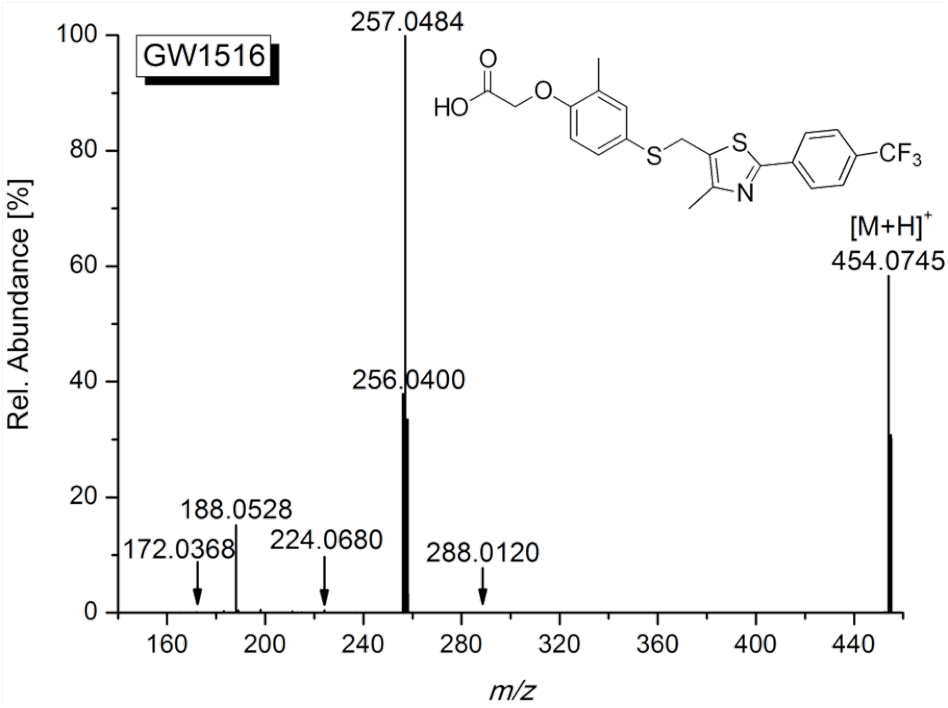

Figure S-4: ESI product ion spectrum of the $[M+H]^+$ ion of PPARδ agonist GW1516 recorded on a QTOF mass spectrometer (TripleTOF™ 5600, Applied Biosystems, Darmstadt, Germany).

Figure S-4 is in agreement with data from Thevis et al. (cf. ESI-MS/MS spectrum in reference 10 [M. Thevis, S. Beuck, A. Thomas, B. Kortner, M. Kohler, G. Rodchenkov, W. Schänzer, *Rapid Commun. Mass Spectrom.* 2009, **23**, 1139-1146]).

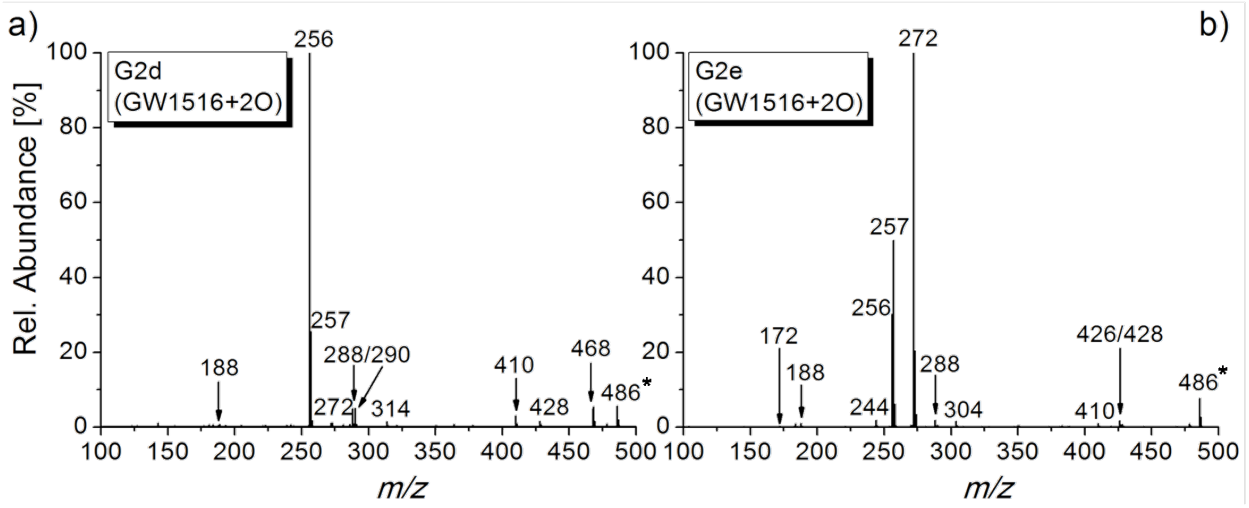

Figure S-5: EPI mass spectra of the $[M+H]^+$ ions (marked with an asterisk) of two isomeric, bis-oxygenated GW1516 metabolites, generated off-line in the EC cell and recorded on a TripleTOF™ 5600 mass spectrometer after LC separation: a) isomer G2d (hydroxylated and *S*-oxidized thiophenol residue), b) isomer G2e (duplicate *S*-oxide).