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**Supplementary data (for Drug Metabolism and Disposition Journal)**

**Utilization of stable isotope labelling to facilitate the identification of polar metabolites of KAF156, an antimalarial agent**

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## Experimental Section

### Materials and Methods

#### Synthesis of fluorophenyl oxoacetic acid

2-(4-fluorophenyl)-2-oxoacetic acid (FPOAA) was synthesized according to the published procedure (Lee and Chen, 1991). 4-fluoromandelic acid (250 mg, 1.5 mmol) was added to 6 mL of a 1.0 M potassium hydroxide solution. The resulting solution was cooled in an ice-water bath and stirred using a magnetic stirring apparatus. A portion of potassium permanganate (474 mg, 3.0 mmol) was added to the solution over 15 min, and the reaction was allowed to proceed for 0.5 h. Sodium sulfite (1.4 g, 11.3 mmol) was added to the reaction, followed by the drop-wise addition of concentrated hydrochloric acid until the mixture turned white. The mixture was warmed to room temperature, and was extracted four times with 50 mL portions of ethyl acetate. The organic layer was separated, dried with anhydrous magnesium sulfate, filtered, and the solvent was removed using a rotary evaporator under vacuum. The organic residue was resuspended in 1 mL of water and acetonitrile was added until the residue fully dissolved (final volume ~2 mL).

The resulting extract was separated using MS-guided fractionation (Waters SQ Detector 2 equipped with: Waters 1525 Binary HPLC Pump, Waters 2767 Sample Manager, and an Agilent Zorbax 5  $\mu$ m RX-C8 9.4 x 250 mm column). The mobile phase was composed of water with 0.05% formic acid (solvent A) and acetonitrile (solvent B). Aliquots (100  $\mu$ L) were injected onto the column followed by a gradient elution (4 mL/min) consisting of an initial 15 min of 2% B, followed by a linear increase to 95% B over 5 min, and finishing with 5 min isocratic elution at 95% B. The MS detector was operated in ESI negative ionization mode. The fraction collector was setup to collect products with  $m/z$  167.1, corresponding to the theoretical  $[M - H]^-$  ion of FPOAA. The collected fractions were combined and the solvent was removed from the sample *via* lyophilization. The resulting organic material was dissolved in DMOS- $d_6$  and pipetted into a 5 mm NMR tube for NMR analysis.

#### Isolation of polar metabolites from rat urine pool

Following oral dosing of a mixture of [ $^{14}$ C]KAF156-2 and [ $^{13}$ C $_2$ ]KAF156, urine samples collected from each rat during 0-24 h postdose were pooled. The urine pool was lyophilized and reconstituted in acetonitrile: deionized water (10/90, v/v). Waters Semi-Preparative LC System and Waters Acquity UPLC system were used, each system equipped with two pumps, an autosampler, a Dual  $\lambda$  Absorbance Detector and a fraction collector.

The separation of polar metabolites was carried out first on a ACE 5AQ (150 x 10.0 mm) semi-prep column and followed by a ACE Excel 2C18 (150 x 4.6 mm) analytical column. The mobile phases consisted of solvent A: water containing 0.1% formic acid; and solvent B: acetonitrile containing 0.1% formic acid. A linear gradient was used and the gradient was initiated at 2%B for 3 min; increased to 30%B from 3 to 28 min; increased to 50%B from 28 to 38 min; increased to 95%B from 38 to 42 min; hold at 95%B from 42 to 46 min. The flow rates were 4 and 0.7 mL/min for semi-prep and analytical column, respectively. Aliquots of the eluents were monitored for radioactivity using a TopCount and confirmed by LC-MS/MS analysis.

The fractions containing polar metabolites were pooled, lyophilized and reconstituted in deuterated solvent (DMSO- $d_6$ , 200  $\mu$ L) for NMR analyses.

## NMR analysis of polar metabolites

**M7:** In preparation for NMR analysis, water was removed from the M7-enriched urine sample by dissolving the sample in DMSO-*d*<sub>6</sub> followed by lyophilization. The dry sample was then dissolved in 200  $\mu$ L DMSO-*d*<sub>6</sub> and pipetted into a 3 mm glass NMR tube. A DQF-COSY spectrum was acquired for the M7 sample using the following parameters: NS = 32, TD = 1024, AQ = 0.75 sec, and the pulse width was calibrated to achieve 90° excitation. The DQF-COSY spectrum was processed using 2K (f1) by 8K (f2) data points and a sine squared window function was applied in both the t1 and t2 dimensions (Figure S6).

A standard addition experiment was carried out wherein 2-(4-fluorophenyl)-2-oxoacetic acid (FPOAA) was added to the *in vivo* derived M7 NMR sample. The M7 sample was prepared as described above, and the FPOAA was prepared by dissolving an unknown amount of FPOAA in 200  $\mu$ L of DMSO-*d*<sub>6</sub>. <sup>1</sup>H NMR spectra were acquired for both the M7 and FPOAA samples using the same acquisition parameters. The resulting spectra were compared by setting the integral value for the area under the CHD<sub>2</sub>SOCD<sub>3</sub> peak (2.50 ppm) equal to 100 and integrating the proton signals corresponding to the protons of the M7/FPOAA aromatic ring (~ 7.88 and 7.25 ppm). After the integrals were compared (~0.2:25, M7:FPOAA, Figure S7 and Figure S8), 10  $\mu$ L of the FPOAA solution was added to 40  $\mu$ L DMSO-*d*<sub>6</sub>. 5  $\mu$ L of the diluted solution was added to the M7 NMR sample, and the sample was inverted several times. A <sup>1</sup>H spectrum was acquired for the mixed M7/FPOAA sample. For the M7, FPOAA, and combined M7/FPOAA samples <sup>1</sup>H spectra were acquired using the following parameters: NS = 4096, AQ = 3.0 sec, and pulse width 7.8  $\mu$ sec. Spectra were processed using 64K zero filling, and the baseline was corrected using Bernstein polynomials (Figure S9).

**M10.1:** The enriched urine sample of M10.1 was dissolved in 200  $\mu$ L of DMSO-*d*<sub>6</sub> and pipetted into a 3 mm glass NMR tube. A DQF-COSY spectrum was acquired for this sample using the following parameters: NS = 32, TD = 1024, AQ = 0.75 seconds, and the pulse width was calibrated to achieve 90° excitation. The DQF-COSY spectrum was processed using 4K data points in f1 and 8K data points in f2. A 90° sine bell window function was applied to the data in both the t1 and t2 dimensions (Figure 10). Chemical shifts were referenced to the residual proton signal of CHD<sub>2</sub>SOCD<sub>3</sub> at 2.50 ppm. A DQF-COSY spectrum was acquired and processed for a standard of M10.1 (4-fluoromandelic acid, obtained from Alfa Aesar) as described above (Figure S10).

**M12:** The enriched urine sample of M12 was dissolved in 200  $\mu$ L of DMSO-*d*<sub>6</sub> and pipetted into a 3 mm glass NMR tube. A <sup>1</sup>H proton spectrum was acquired, baseline corrected using Bernstein polynomials, and manually integrated (Figure S11). A DQF-COSY spectrum was acquired for this sample using the following parameters: NS = 64, TD = 1024, AQ = 0.75 sec, and the pulse width was calibrated to achieve a 90° excitation. The DQF-COSY spectrum was processed using 4K (f1) by 8K (f2) data points. A 90° sine bell window function was applied in t2 and a 90° sine squared window function was applied in t1 (Figure S12). A ROESY spectrum was acquired for the M12 sample using the following parameters: NS = 64, TD = 256, AQ = 0.25 seconds, and a mixing time of 300 msec. The ROESY spectrum was processed using 4K (f1) by 4K (f2) data points. The spectrum was baseline corrected using Bernstein polynomials. A 90° sine squared window function was applied in t1, and a 0.10 Hz exponential window function was applied in t2 (Figure S13) For all the M12 spectra, chemical shifts were referenced to the residual proton signal of CHD<sub>2</sub>SOCD<sub>3</sub> at 2.50 ppm.

## Results

### NMR analysis

**M7:** In order to support the structural proposal for M7 obtained from mass spectrometric analyses, M7 was probed using NMR spectroscopy. The comparison of  $^1\text{H}$  spectra acquired for the rat urine-derived M7-enriched sample and the FPOAA standard showed a slight difference in the chemical shifts of the aromatic protons (Figure S7 and Figure S8), potentially caused by differences in sample concentrations and/or pHs. Therefore a standard addition experiment was performed in which the FPOAA standard was added to the M7 sample to achieve an approximate 1:1 molar ratio between the putative M7 analyte and FPOAA. As shown in Figure S9 the addition of FPOAA increased the area under the indicated aromatic peak by approximately 2-fold, whereas area under the surrounding signals remained constant. These data demonstrate that the original M7 sample contained FPOAA.

**M10.1:** The DQF-COSY spectrum corresponding to the M10.1-enriched sample showed a pair of cross peaks in the aromatic region with atypical coupling patterns (a typical DQF-COSY peak showing complete anti-symmetry when bisected and compared in both the vertical and horizontal dimensions). The pattern of these cross peaks suggested the presence of an aryl group substituted with a fluorine atom at the para position (Figure S10). Comparison of the DQF-COSY acquired for the M10.1 sample with the DQF-COSY spectrum acquired for the 4-fluoromandelic acid standard showed matching chemical shifts and coupling patterns, supporting the M10.1 structural proposal generated from mass spectrometric analysis.

**M12:** The  $^1\text{H}$  spectrum acquired for the M12-enriched NMR sample, derived from rat urine, showed two signals shifted downfield that integrate with whole-number ratios relative to each other (7.93 and 8.66 ppm; relative integration, 2:1; Figure S11). The chemical shift and relative integration of the signal at 8.66 ppm suggested that this peak represents an amide proton. A DQF-COSY spectrum acquired for the sample revealed that the putative amide proton couples to a methylene at 3.82 ppm (Figure S12). Additionally, the DQF-COSY coupling patterns observed for the aromatic protons at 7.93 ppm were indicative of an aryl group with a fluorine atom substituted at the para position. Further analysis *via* ROESY acquisition, an experiment that shows  $^1\text{H}, ^1\text{H}$ -coupling through space, demonstrated that the amide proton is proximal to the aromatic protons observed at 7.93 ppm (Figure S13). The chemical shift and position of the methylene, distal to the aromatic ring, was consistent with it being positioned between the amide nitrogen and a carboxylic acid group. This hypothesis was supported by mass spectrometric analysis and comparison of observed NMR chemical shifts to those reported for 4-fluoro-hippuric acid (Chaves, 2010)

**Table S1 Summary of mass balance of [<sup>14</sup>C]KAF156-1 and [<sup>14</sup>C]KAF156-2 in rats**

Urine and feces samples were collected daily for up to 7 days from rats. Aliquots of urine samples were counted directly for radioactivity. After homogenization, aliquots of fecal homogenate were solubilized and counted for radioactivity. The recoveries of radioactivity in excreta were expressed as % of dose by collection time intervals. Total mean recovery ranging from ~93-118% was within experimental error.

<sup>14</sup> C]KAF156-1								
Dosing Route	IV (3 mg/kg)				PO (10 mg/kg)			
Matrices	Urine		Feces		Urine		Feces	
Interval (h)	Mean	SD	Mean	SD	Mean	SD	Mean	SD
0-28	38.8	4.75	43.5	8.19	30.4	2.62	51.6	3.61
28-48	9.6	1.73	12.8	5.40	5.10	0.429	9.33	2.40
48-72	3.8	0.18	2.40	1.14	2.50	0.348	1.36	0.190
72-96	1.6	0.48	0.893	0.160	0.997	0.094	0.394	0.070
96-168	1.9	0.65	0.988	0.150	1.17	0.148	0.633	0.170
Subtotal	55.7	5.48	60.6	5.11	40.2	2.18	63.3	1.28
Cage wash			2.07	0.430			1.89	1.43
Total			118	4.66			105	4.84
<sup>14</sup> C]KAF156-2								
Dosing Route	IV (3 mg/kg)				PO (10 mg/kg)			
Matrices	Urine		Feces		Urine		Feces	
Interval (h)	Mean	SD	Mean	SD	Mean	SD	Mean	SD
0-24	16.1	2.29	41.3	9.02	16.0	11.8	46.7	5.81
24-48	3.76	1.30	21.7	7.81	3.41	0.919	23.9	8.25
48-72	1.23	0.376	2.94	0.430	1.87	0.734	2.21	0.578
72-96	1.08	0.512	0.802	0.254	1.29	0.553	0.90	0.673
96-168	1.03	0.497	1.21	0.386	0.976	0.423	0.963	0.539
Subtotal	23.2	1.75	68.0	5.59	23.5	11.2	74.7	4.35
Cage wash			2.13	1.00			1.31	1.35
Total			93.3	5.36			99.6	6.16

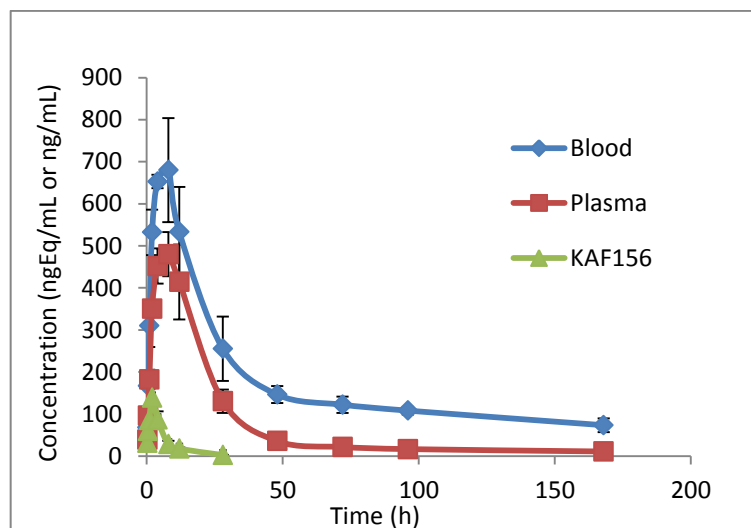
**Table S2      Pharmacokinetics of KAF156 in rat plasma**

Blood samples were collected at selected time intervals from each animal (n=3 by either dosing route). Plasma samples were prepared by centrifugation. After extraction, plasma samples were analysed by LC-MS/MS analysis. Pharmacokinetics parameters were calculated using Phoenix (WinNonlin Version 6.2).

<i>i.v.</i> dose (mg/kg)	3		
AUC (ng.h/mL)	556	±	19.1
CL (L/h/kg)	5.4	±	0.189
V <sub>dss</sub> (L/kg)	29.5	±	1.15
t <sub>1/2</sub> (h)	6.6	±	0.6
<i>p.o.</i> dose (mg/kg)	10		
C <sub>max</sub> (ng/mL)	139	±	27
T <sub>max</sub> (h)	2	±	0
AUC (ng.h/mL)	887	±	136
t <sub>1/2</sub> (h)	5.7	±	0.3
%F	47.9	±	7.35

**Figure S1** Mean concentrations of KAF156 and total radioactivity in blood and plasma from rat following oral dosing of [ $^{14}\text{C}$ ]KAF156-1

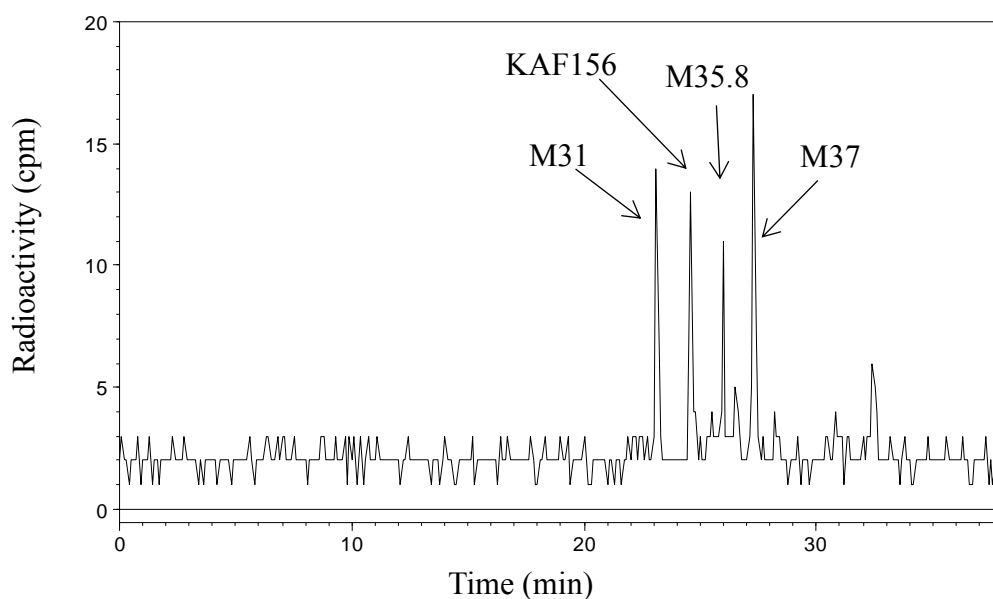
Blood samples were collected at selected time intervals from rats. Plasma samples were prepared by centrifugation. Concentrations of radioactivity in blood and plasma were determined by scintillation counting and concentrations of KAF156 were determined by LC-MS/MS analysis. The concentrations of  $^{14}\text{C}$  in blood,  $^{14}\text{C}$  in plasma and KAF156 in plasma were plotted against time. Pharmacokinetics parameters of KAF156 were determined and described in Table 2.



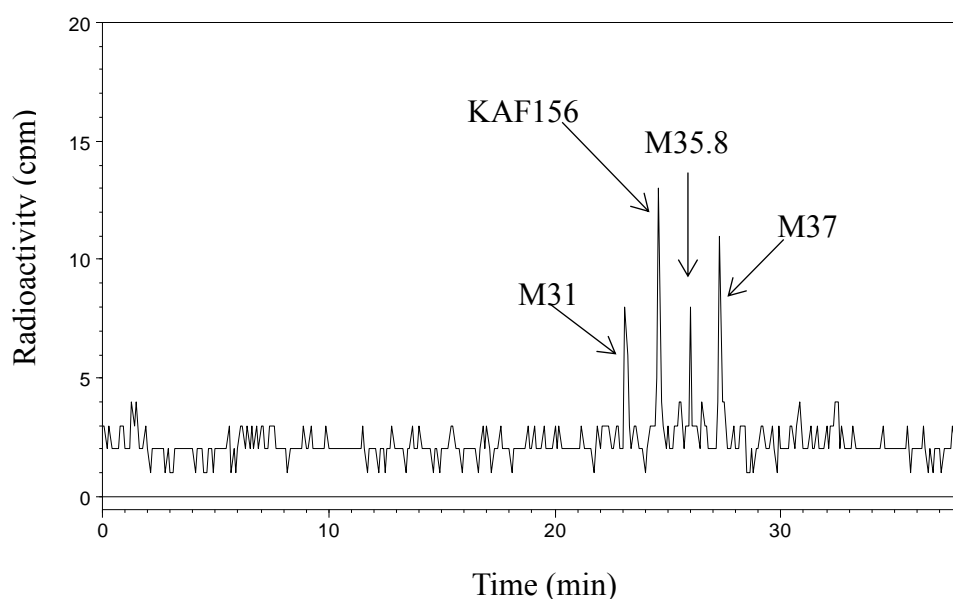
**Figure S2** Representative metabolic profiles in plasma from rats following (A) *i.v.* or (B) *p.o.* dosing of [<sup>14</sup>C]KAF156-2

Blood samples were collected at selected time intervals from rats. After centrifugation, plasma samples were pooled from each animal (n=3) from the same collection time. The pooled plasma samples were extracted and analyzed by LC-MS/MS. The HPLC separation of KAF156-2 and metabolites was performed using a Zorbax SB-C18 column, as described in *Materials and Methods*. All metabolites were designated by a letter M followed by their approximate HPLC retention times in the study in which they were first observed. The structural assignments were proposed based on LC-MS/MS analysis.

(A) *i.v.* 4 h postdose



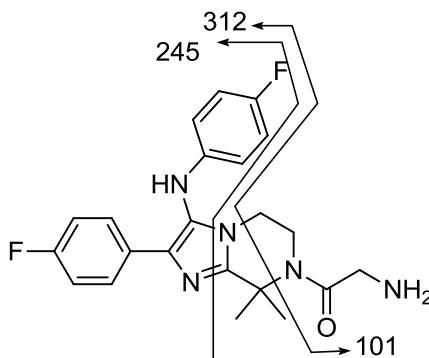
(B) *p.o.* 4 h postdose



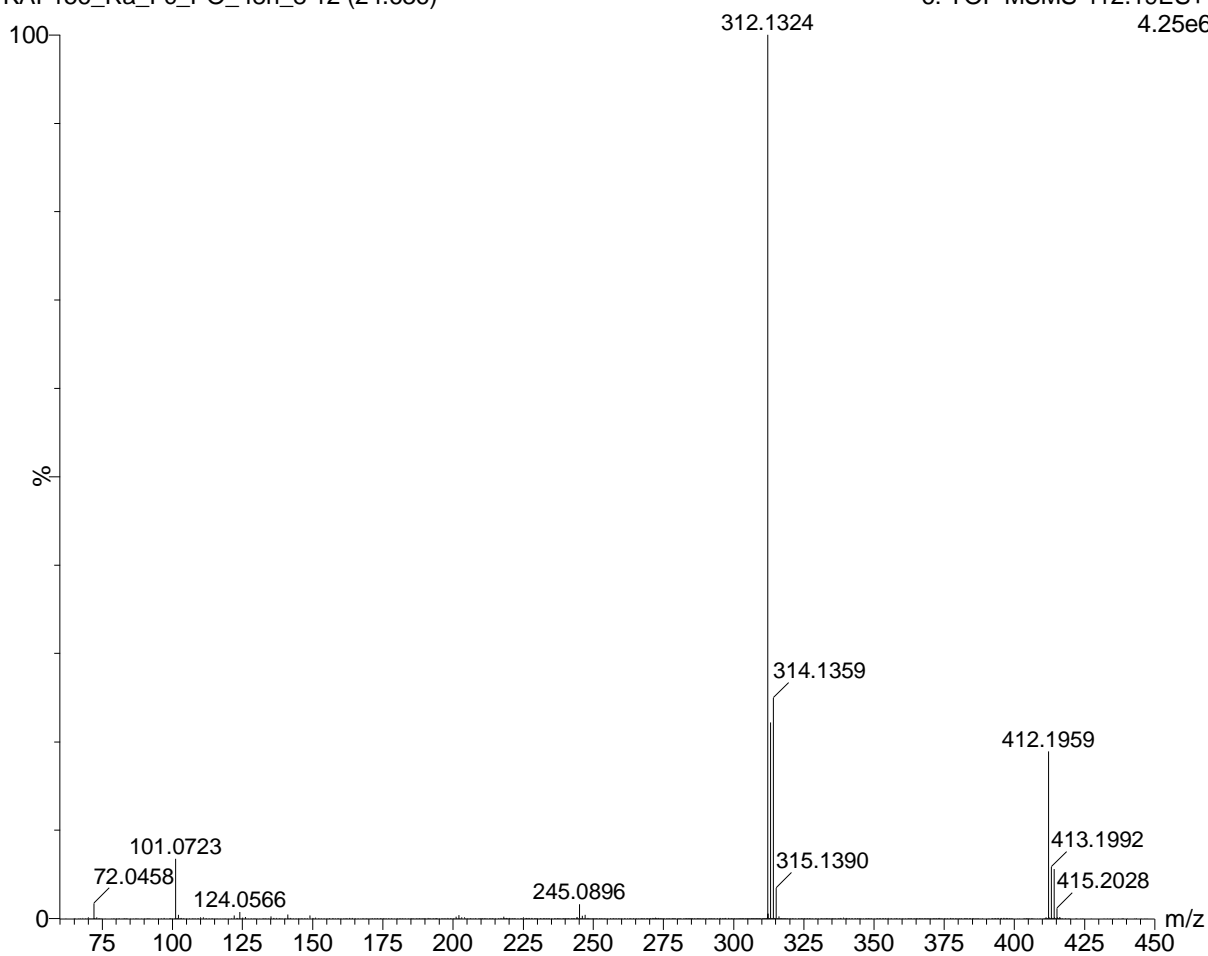


**Figure S3**                      **A representative product ion spectrum of KAF156**

Rat fecal samples were extracted and analysed by LC-MS/MS under positive ion mode, as described in *Materials and Methods*. The resulting fragmentation ions of KAF156 are illustrated.

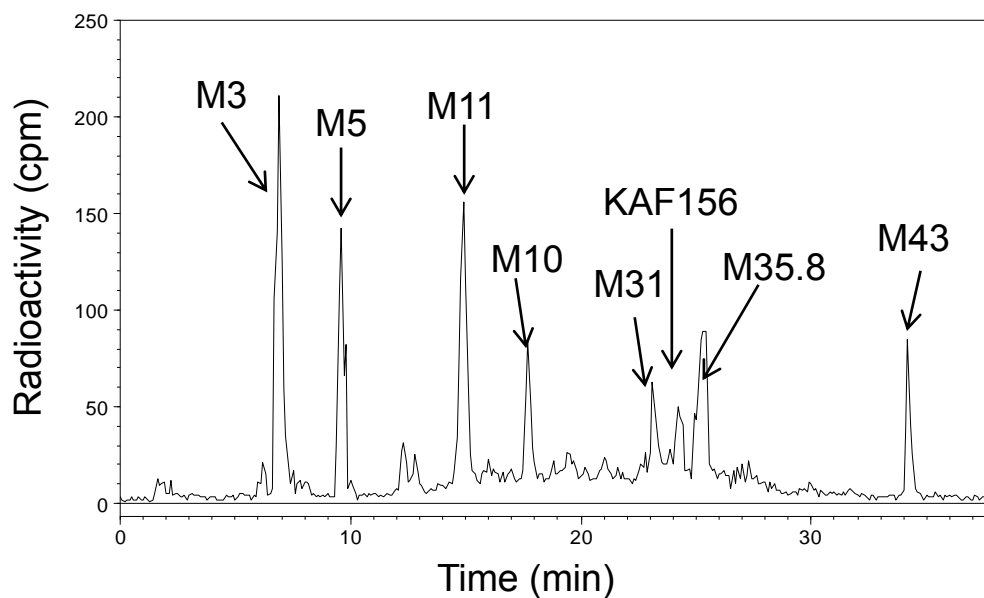
**MSMS, KAF156, Rat Feces PO 48h, Positive**

KAF156\_Ra\_Fc\_PO\_48h\_8 12 (24.636)

6: TOF MSMS 412.19ES+  
4.25e6

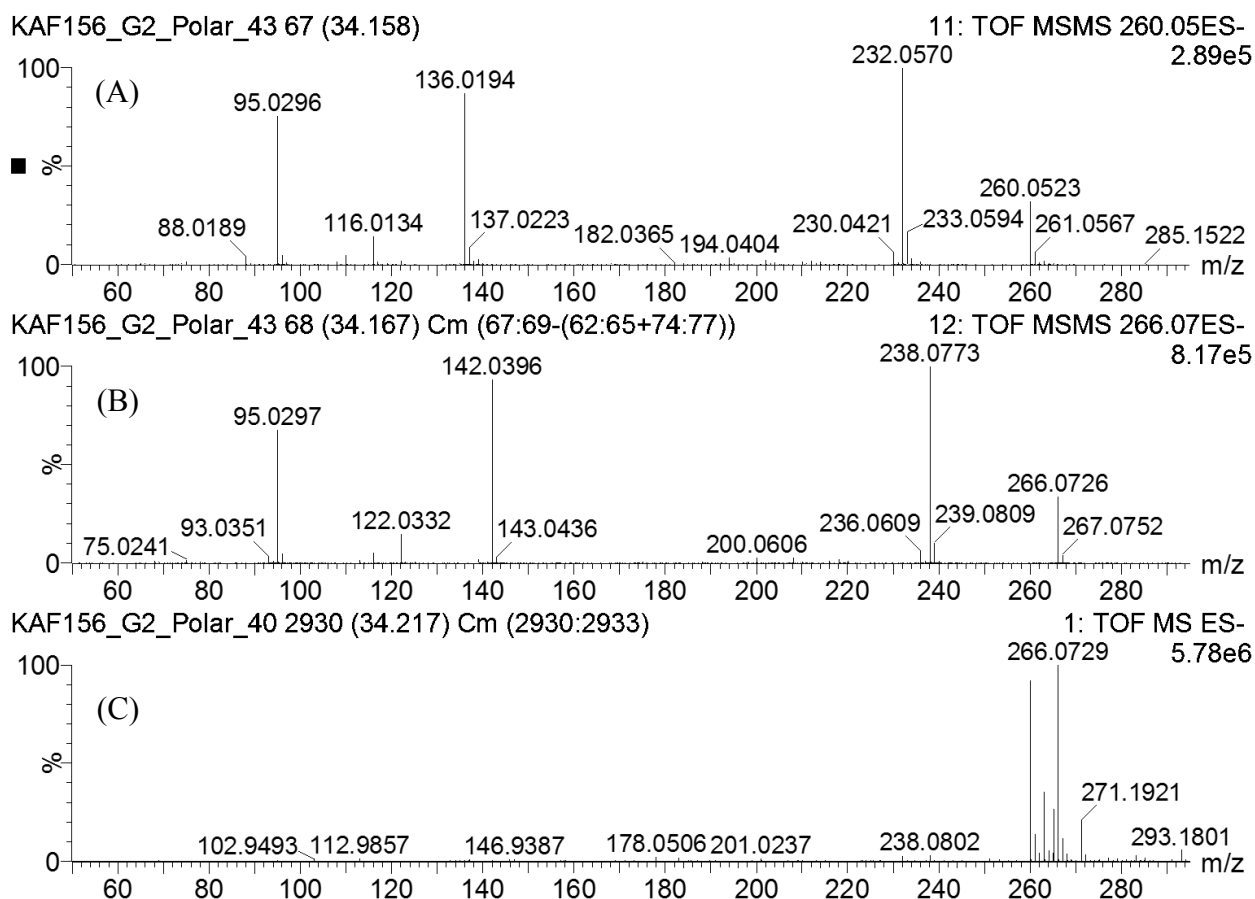
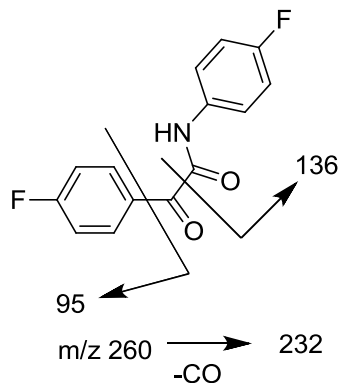
**Figure S4 A representative metabolic profile of polar metabolites in rat urine**

A new UPLC method was developed in order to retain the polar metabolites, as described in material and methods (method B). The polar metabolites were identified by LC-MS/MS under negative ion mode. The assignments of metabolites were based on comparison of retention time of two UPLC systems and LC-MS/MS data. M10 was eluted after M11 in this system (method B) while M10 was eluted before M11 using method A (Figure 2 in manuscript).

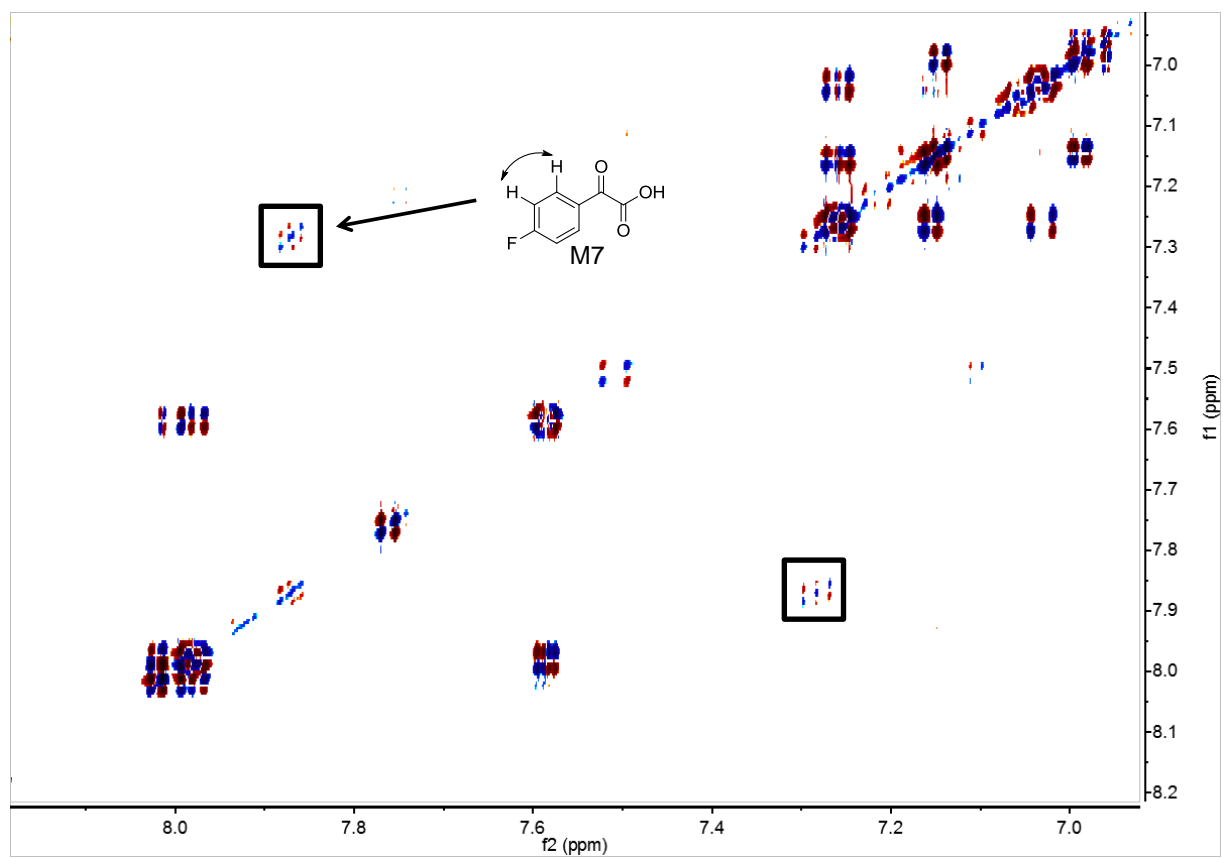


**Figure S5 A representative product ion spectrum of M43**

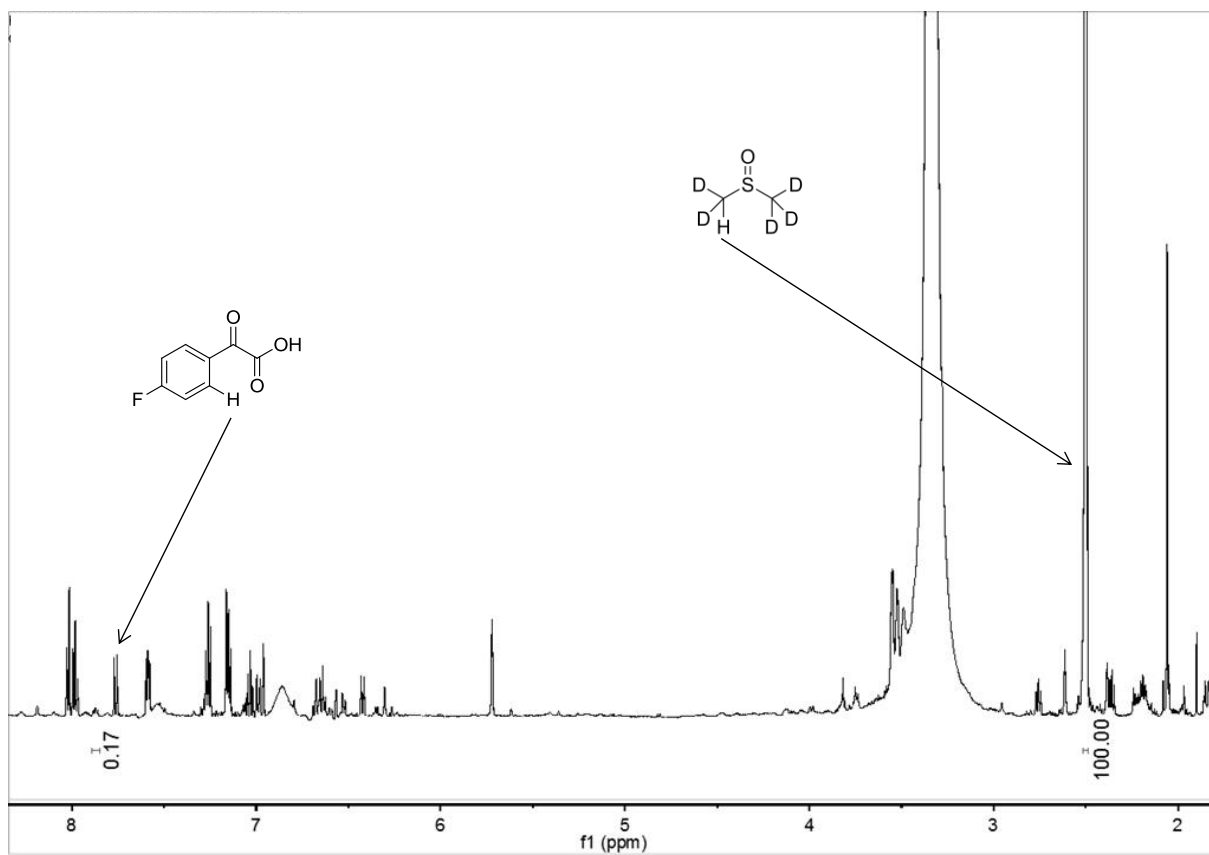
Rat urine samples were centrifuged and analysed by LC-MS/MS under negative ion mode, as described in *Materials and Methods*. The resulting fragmentation ions of M43 are illustrated.



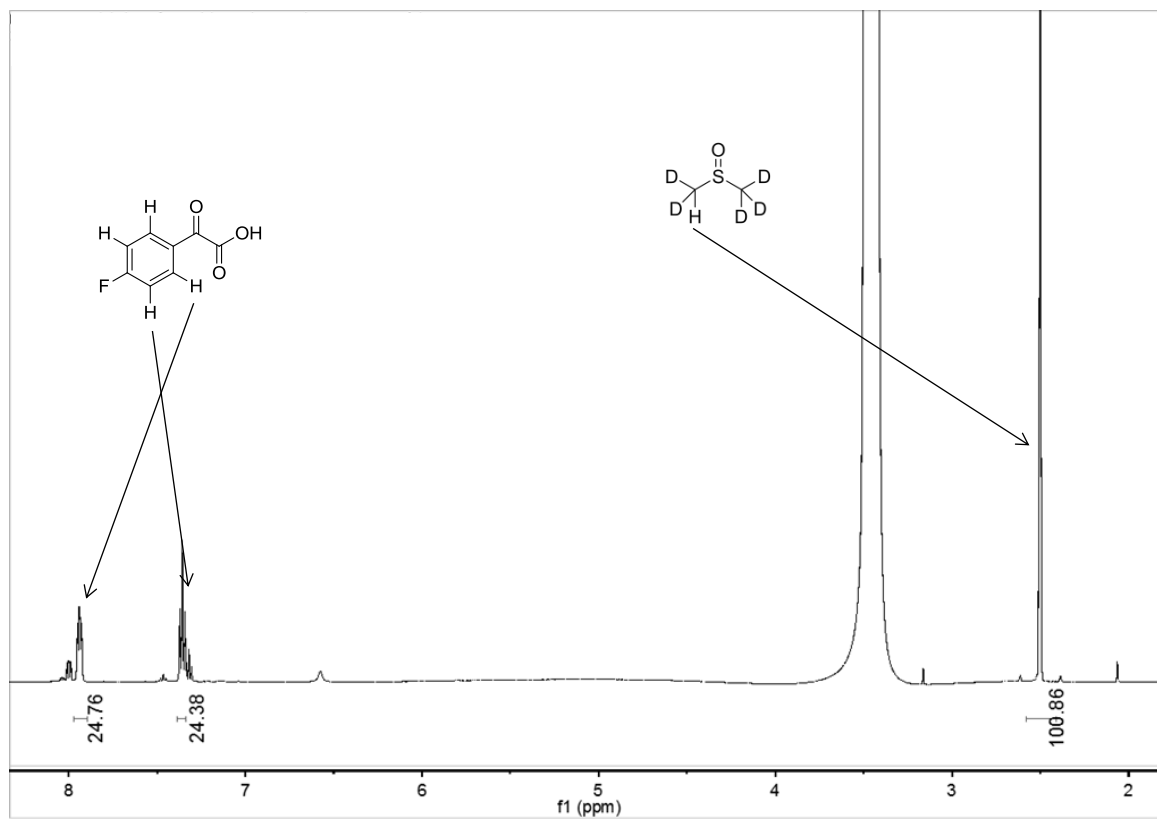
**Figure S6** Aromatic region of the DQF-COSY spectrum acquired for the M7-enriched urine sample



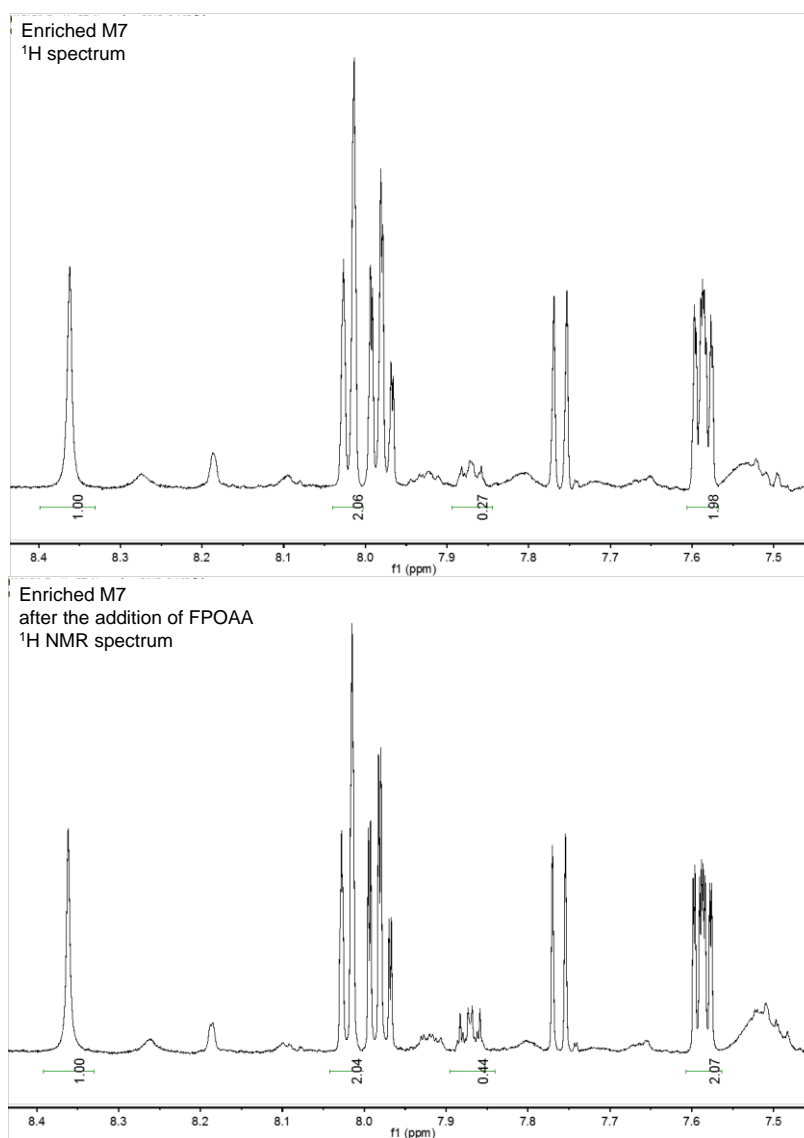
**Figure S7**  $^1\text{H}$  spectrum of the enriched M7-containing urine sample with the M7 aromatic peaks integrated relative to the  $\text{CHD}_2\text{SOCD}_3$  signal, 600 MHz NMR spectrometer



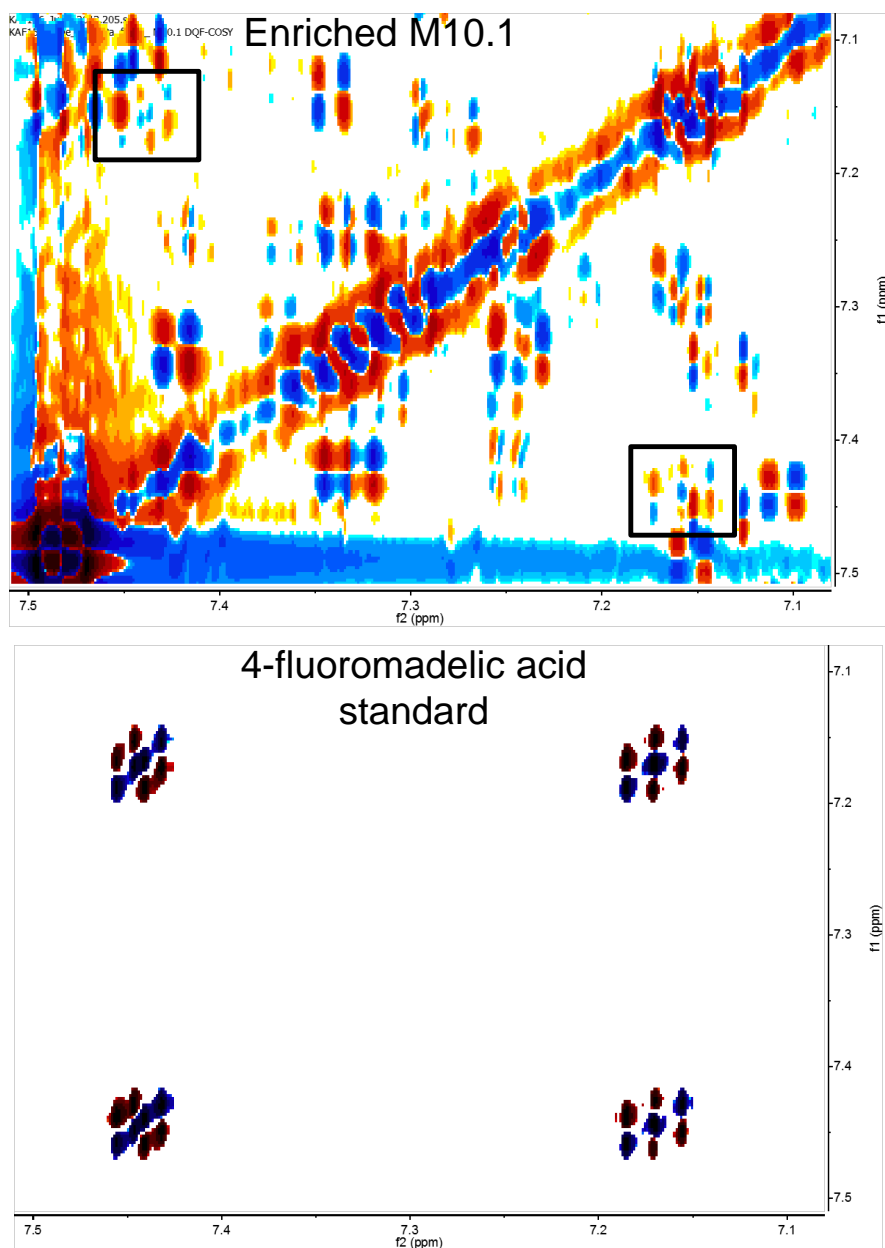
**Figure S8**  $^1\text{H}$  spectrum of 2-(4-fluorophenyl)-2-oxoacetic acid (FPOAA) with the FPOAA aromatic peaks integrated relative to the  $\text{CHD}_2\text{SOCD}_3$  signal, 600 MHz NMR spectrometer



**Figure S9** The aromatic region of the  $^1\text{H}$  spectra corresponding to the urine-derived enriched M7 NMR sample and, for the same sample, after the addition of the 2-(4-fluorophenyl)-2-oxoacetic acid standard

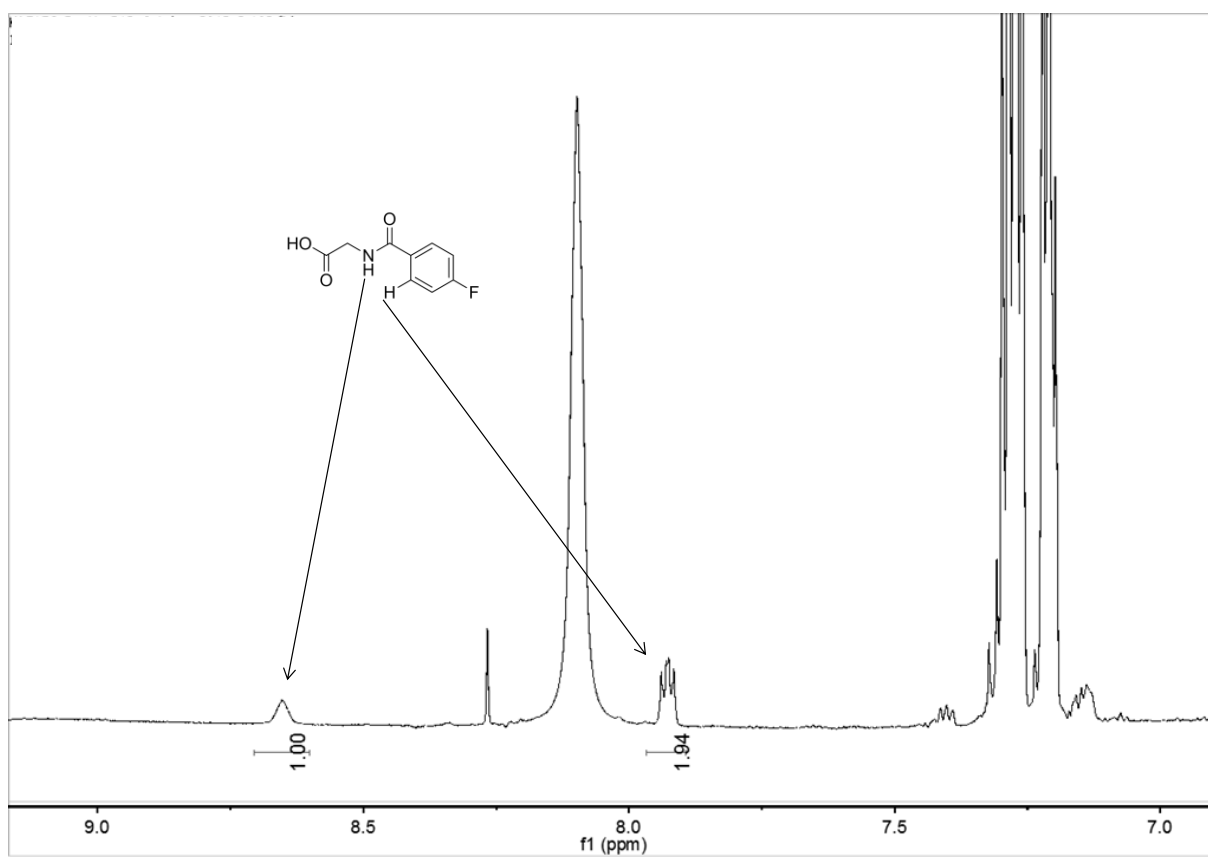


**Figure S10** Aromatic region of the DQF-COSY spectra corresponding to the enriched M10.1 NMR sample derived from rat urine and the standard 4-fluoromandelic acid, DMSO-*d*<sub>6</sub>, 600 MHz NMR spectrometer

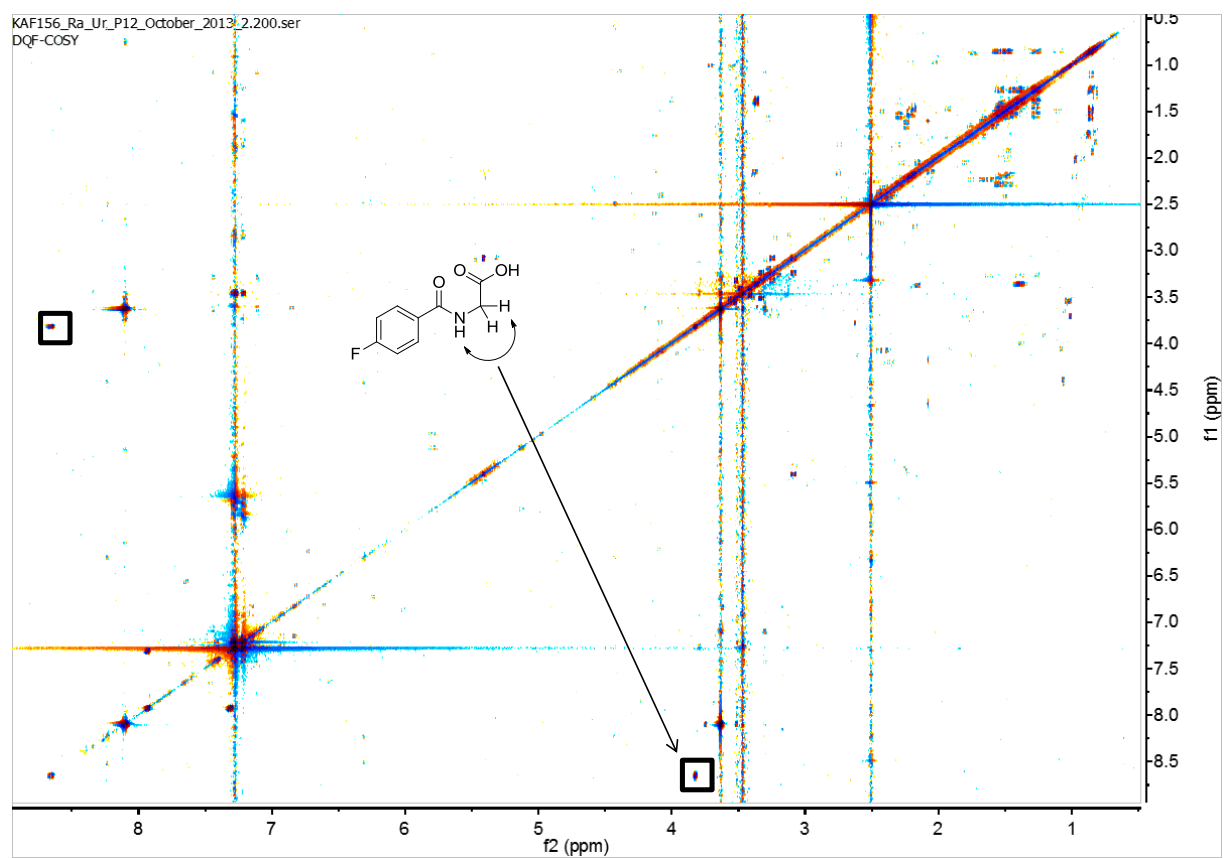




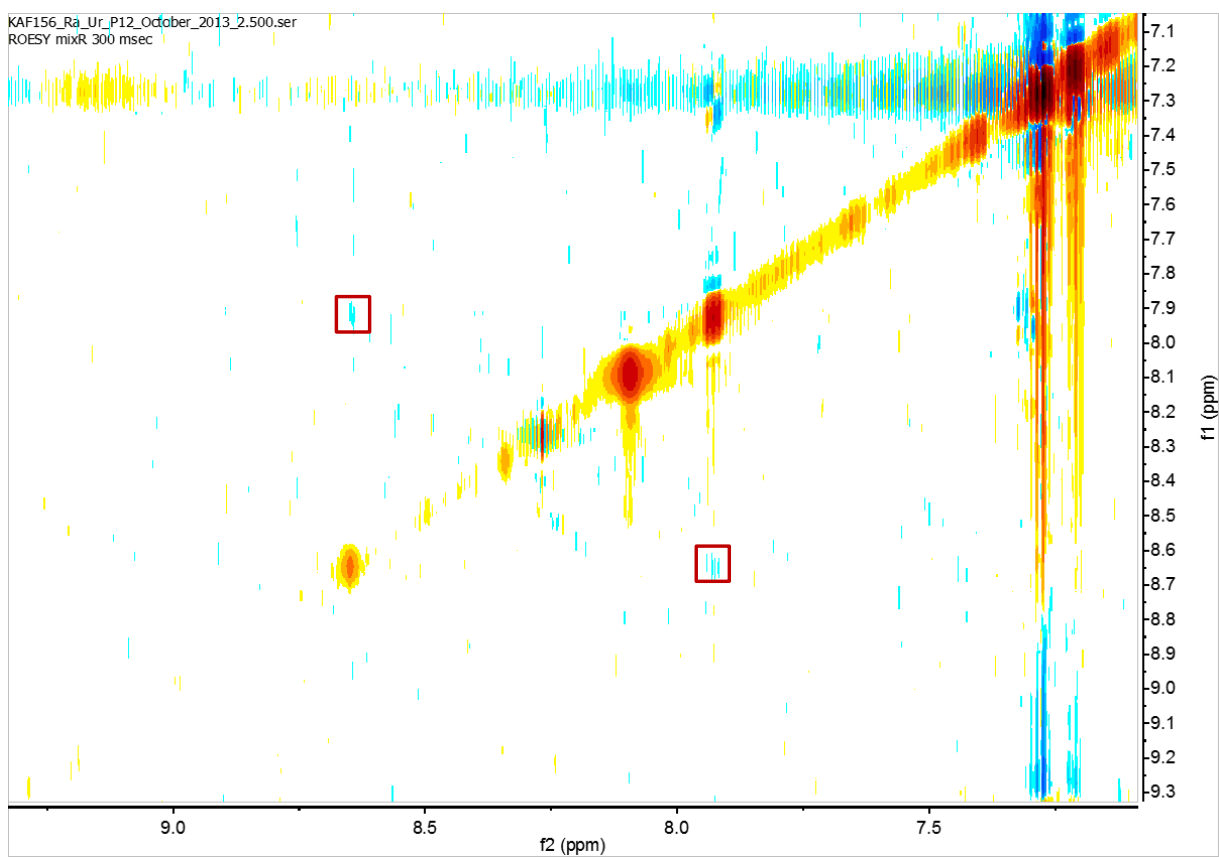
**Figure S11** Aromatic region of the  $^1\text{H}$  spectrum acquired for the M12 NMR sample enriched from rat urine,  $\text{DMSO-}d_6$ , 600 MHz NMR spectrometer



**Figure S12** DQF-COSY spectrum M12 NMR sample enriched from rat urine, boxes indicate coupling between the amide and methylene protons, DMSO-*d*<sub>6</sub>, 600 MHz NMR spectrometer

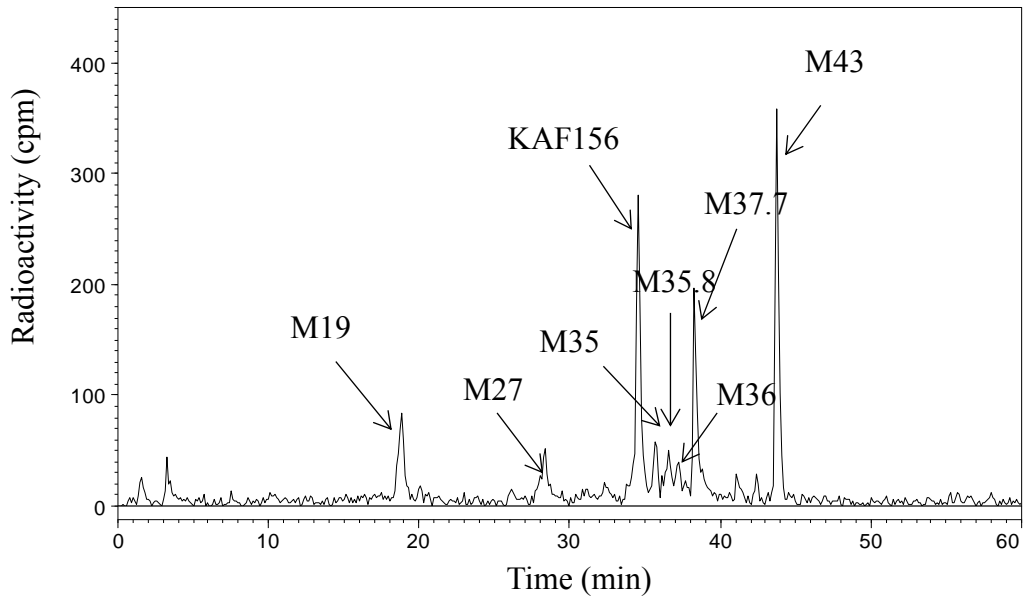


**Figure S13** Aromatic region of the ROESY spectrum acquired for the enriched M12 NMR sample derived from rat urine, highlighted cross peaks indicated coupling between amide and aromatic protons, DMSO-*d*<sub>6</sub>, 600 MHz NMR spectrometer



**Figure S14** Representative metabolic profiles of [ $^{14}\text{C}$ ]KAF156-1 in (A) rat and (B) human hepatocytes

(A)



(B)

