Wagmann, L, Hempel, N, Richter, LHJ, Brandt, SD, Stratford, A and Meyer, MR

Phenethylamine-derived new psychoactive substances 2C-E-FLY, 2C-EF-FLY, and 2C-T-7-FLY: Investigations on their metabolic fate including isoenzyme activities and their toxicological detectability in urine screenings

http://researchonline.ljmu.ac.uk/id/eprint/11014/

Article
Phenethylamine-derived new psychoactive substances 2C-E-FLY, 2C-EF-FLY, and 2C-T-7-FLY: Investigations on their metabolic fate including isoenzyme activities and their toxicological detectability in urine screenings

Lea Wagmann¹ | Nora Hempel¹ | Lilian H.J. Richter¹ | Simon D. Brandt² | Alexander Stratford³ | Markus R. Meyer¹

¹ Department of Experimental and Clinical Toxicology, Institute of Experimental and Clinical Pharmacology and Toxicology, Center for Molecular Signaling (PZMS), Saarland University, Homburg, Germany
² School of Pharmacy and Biomolecular Sciences, Liverpool John Moores University, Liverpool, UK
³ Synex Synthetics BV, Karveelweg 20, 6222NH, Maastricht, Netherlands

Correspondence
Markus R. Meyer, Department of Experimental and Clinical Toxicology, Institute of Experimental and Clinical Pharmacology and Toxicology, Center for Molecular Signaling (PZMS), Kirrberger Str. Geb. 46, Saarland University, 66421, Homburg, Germany
Email: markus.meyer@uks.eu

Funding information
Armin A. Weber; Hans H. Maurer; Gabriele Ulrich

Abstract
Psychoactive substances of the 2C-series are phenethylamine-based designer drugs that can induce psychostimulant and hallucinogenic effects. The so-called 2C-FLY series contains rigidified methoxy groups integrated in a 2,3,6,7-tetrahydrobenzo[1,2-b:4,5-b']difuran core. The aim of the presented work was to investigate the in vivo and in vitro metabolic fate including isoenzyme activities and toxicological detectability of the three new psychoactive substances (NPS) 2C-E-FLY, 2C-EF-FLY, and 2C-T-7-FLY to allow clinical and forensic toxicologists the identification of these novel compounds. Rat urine, after oral administration, and pooled human liver S9 fraction (pS9) incubations were analyzed by liquid chromatography–high-resolution tandem mass spectrometry (LC–HRMS/MS). By performing activity screenings, the human isoenzymes involved were identified and toxicological detectability in rat urine investigated using standard urine screening approaches (SUSAs) based on gas chromatography (GC)–MS, LC–MSn, and LC–HRMS/MS. In total, 32 metabolites were tentatively identified. Main metabolic steps consisted of hydroxylation and N-acetylation. Phase I metabolic reactions were catalyzed by CYP2D6, 3A4, and FMO3 and N-acetylation by NAT1 and NAT2. Methoxyamine was used as a trapping agent for detection of the deaminated metabolite formed by MAO-A and B. Interindividual differences in the metabolism of the 2C-FLY drugs could be caused by polymorphisms of enzymes involved or drug–drug interactions. All three SUSAs were shown to be suitable to detect an intake of these NPS but common metabolites of 2C-E-FLY and 2C-EF-FLY have to be considered during interpretation of analytical findings.

KEYWORDS
drugs of abuse, LC–HRMS/MS, metabolism, new psychoactive substances
Innumerable new psychoactive substances (NPS) are sold as drugs of abuse. NPS are often derivatives of drugs under legislative control mimicking their psychoactive effects. This is also true for the 2C-FLY drugs, which are derived from the well-known phenethylamines of the 2C-type and which are also expected to induce psychostimulant and hallucinogenic effects. Chemically, they contain rigidified methoxy groups integrated in a 2,3,6,7-tetrahydrofuro[2,3-b]furan core in contrast to the classic 2Cs with two methoxy groups located in positions 2 and 5 of the phenyl ring. Exposure to NPS can result in significant health risks as demonstrated by frequent reports on toxic effects and fatal intoxications. Therefore, clinical and forensic toxicologists should be able to reliably detect an intake of NPS. Both parameters and metabolites can be suitable screening targets in development of urine screening approaches. A prerequisite is knowledge of their toxicokinetic. Furthermore, knowledge of the isoenzymes involved in the metabolism is important to predict drug–drug/drug–food interactions and/or individual differences found in half-life and excretion patterns.

A series of FLY compounds were originally synthesized to study 5-HT2A receptor function and 2-(4-bromo-2,3,6,7-tetrahydrofuro[2,3-f] [1]benzofuran-8-yl)ethanamine (2C-B-FLY) was shown to potently bind to the human 5-HT2A receptor. 2C-B-FLY was first reported to the early warning system on NPS implemented by the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) in 2007 and several times afterwards. However, little is known about the toxicokinetics of 2C-FLY derivatives. Recently, the four 2C-FLY compounds 2C-B-FLY, 2-(4-ethyl-2,3,6,7-tetrahydrofuro[2,3-f] [1]benzofuran-8-yl)ethanamine (2C-E-FLY), 2-(4-iido-2,3,6,7-tetrahydrofuro[2,3-f] [1]benzofuran-8-yl)ethanamine (2C-I-FLY), and 2-(4-propylthio-2,3,6,7-tetrahydrofuro[2,3-f] [1]benzofuran-8-yl)ethanamine (2C-T-FLY) were identified as inhibitors of the monoamine oxidase A. An in vitro metabolism study of 2C-B-FLY was also published recently, but nothing is known about the toxicokinetics of the other 2C-FLY compounds.

Therefore, the aim of the presented work was to close this gap by investigating the in vivo and in vitro metabolic fate of 2C-E-FLY, 2-(4-(2-fluoro)ethyl-2,3,6,7-tetrahydrofuro[2,3-f] [1]benzofuran-8-yl)ethanamine (2C-EF-FLY), and 2C-T-7-FLY (Figure 1) followed by urinary detectability studies and identification of enzymes involved in initial metabolic steps.

![FIGURE 1](image-url) Chemical structures of the investigated 2C-FLY drugs

## 2 EXPERIMENTAL

### 2.1 Chemicals and enzymes

2C-E-FLY, 2C-EF-FLY, and 2C-T-7-FLY were provided as hydrochloride salts by Synex Synthetics BV (Maastricht, Netherlands). Isocitrate, isocitrate dehydrogenase (IDH), superoxide dismutase (SOD), 3′-phosphoadenosine-5′-phosphosulfate (PAPS), 5′-(5′-adenosyl)-L-methionine (SAM), dithiothreitol (DTT), reduced glutathione (GSH), acetylcarnitine transferase (AcCT), acetylcarnitine, acetyl coenzyme A (AcCoA), potassium dihydrogenphosphate, dipotassium hydrogenphosphate, Tris base, triethanolamine (TEA), ethylenediaminetetraacetic acid, and methoxyamine were obtained from Sigma-Aldrich (Taufkirchen, Germany) and NADP* from Biomol (Hamburg, Germany). Acetonitrile (ACN, LC-MS grade), ammonium formate (analytical grade), formic acid (LC-MS grade), methanol (LC-MS grade), and all other chemicals and reagents (analytical grade) were obtained from VWR (Darmstadt, Germany).

The baculovirus-infected insect cell microsomes (Supersomes) containing the human complementary cDNA-expressed cytochrome P450 (CYP) enzymes CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP3A4 (1 nmol/mL), or CYP2E1, CYP3A5 (2 nmol/mL), or flavin-containing monoxygenase 3 (FMO3), MAO-A, or MAO-B (5 mg protein/mL), or wild type Supersomes without MAO activity as negative control (MAO control, 5 mg protein/mL), and pooled human liver microsomes (pHLMs, 20 mg microsomal protein/mL, 330 pmol total CYP/mg protein), pS9 (20 mg microsomal protein/mL), uridine 5′-diphospho-glucuronosyltransferase (UGT) reaction mixture solution A (25mM UDP-glucuronic acid), and UGT reaction mixture solution B (250mM Tris–HCl, 40mM MgCl₂, and 0.125 mg/mL alamethicin) were obtained from Corning (Amsterdam, Netherlands). Supersomes containing human complementary cDNA-expressed NAT1 (arylamine NAT1*4, wild-type allele) and NAT2 (arylamine NAT2*4 wild-type allele) were obtained from BD Biosciences (Heidelberg, Germany). After delivery, the enzyme preparations and PAPS were thawed at 37°C, aliquoted, snap-frozen in liquid nitrogen, and stored at −80°C until use.

### 2.2 Rat urine samples

As already described in previous studies, the in vivo experiments were performed using urine samples from male Wistar rats (Charles River, Sulzfeld, Germany) for toxicological diagnostic reasons according to German law ("Tierschutzgesetz", Bundesministerium der Justiz und für Verbraucherschutz, 2018, dealing with the treatment of laboratory animals and the experimental design aiming to prevent unnecessary pain, harm, and distress). First, blank urines were collected over a period of 24 hours to verify the absence of interfering compounds. Afterwards the compounds were administered in an aqueous solution via gastric intubation as a single dose of 0.2 mg/kg body weight (BW) for toxicological detectability studies (low dose) and a single dose of 2 mg/kg BW (high dose) for identification of the metabolites and toxicological detectability studies. During the collection of urine over a
period of 24 hours, the rats were housed in metabolism cages and urine was collected separated from feces. The urine samples were analyzed directly and remaining samples were aliquoted and stored at −20°C. One rat was used per NPS.

2.3 | Rat urine sample preparation for identification of metabolites

2.3.1 | Precipitation

Identification of phase I and II metabolites in rat urine was performed after urine precipitation (UP) as described elsewhere. A volume of 100 μL urine collected after high dose administration was precipitated with 500 μL acetonitrile. The mixture was vortexed and centrifuged for 2 minutes at 18,407 x g. The supernatant was evaporated to dryness under a stream of nitrogen. The residue was reconstituted in 50 μL of a 1:1 (v/v) mixture of eluents A and B and transferred to an autosampler vial. One μL was injected onto the LC−HRMS/MS system.

2.3.2 | Solid-phase extraction

According to Maurer et al., 2.5 mL of high dose rat urine was poured into a centrifugal glass and 2 mL of distilled water and 50 μL of 0.01 mg/mL trimipramine-d3 as internal standard were added. The HCX cartridge was conditioned with 1 mL methanol and 1 mL distilled water before transferring the samples to the cartridge. After the passage of the sample, the cartridge was washed with 1 mL distilled water, 1 mL 0.01 M hydrochloric acid, and 2 mL methanol. Thereafter, compounds of interest were eluted with 1 mL of a mixture of methanol/aqueous ammonia 33% (98:2, v/v) into an autosampler vial and evaporated to dryness under a stream of nitrogen at 60°C. The extract was reconstituted in 50 μL methanol and 1 μL was injected onto the LC−HRMS/MS system.

2.4 | pS9 incubations

As described before, the final incubation volume was 150 μL and the final protein concentration was 2 mg/mL. All given concentrations are final concentrations in the incubation mixture. First, a mixture containing 25 μg/mL alamethicin (UGT reaction mixture solution B), 90 mM phosphate buffer (pH 7.4), 2.5 mM Mg2+, 2.5 mM isocitrate, 0.6 mM NADP+, 0.8 U/mL IDH, 100 U/mL SOD, 0.1 mM AcCoA, 2.3 mM acetyl carnitine, 8 μM AcT, and 2 mg/mL pS9 was preincubated for 10 minutes at 37°C. Afterwards, 2.5 mM UDP glucuronic acid (UGT reaction mix solution A), 40 μM aqueous PAPS, 1.2 mM SAM, 1 mM DTT, and 10 mM GSH were added. The reactions were started by addition of 25 μM of one of the 2C-FLY derivatives in phosphate buffer and the tube was incubated for 6 hours. After 1 hour, 60 μL of the incubation mixture was transferred into another tube and the reaction was terminated by addition of 20 μL ice-cold acetonitrile. The remaining mixture (90 μL) was incubated for an additional 5 hours and then stopped with 30 μL ice-cold acetonitrile. All solutions were cooled for 30 minutes at −20°C, centrifuged for 2 minutes at 18,407 x g, and 50 μL of the supernatants were transferred to autosampler vials. One μL was injected onto the LC−HRMS/MS system. Blank incubations without 2C-FLY derivative and control samples without pS9 were prepared to confirm the absence of interfering compounds and to identify compounds that are not of metabolic origin. All incubations were done in duplicate.

2.5 | Isoenzyme activity screenings

2.5.1 | Monooxygenases activity screening

The microsomal incubations were performed at 37°C for 30 minutes with 25 μM of the 2C-FLY analog and 50 pmol/mL CYP isoenzyme (CYP1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, 3A4, or 3A5) or 0.25 mg protein/mL FMO3. The incubation mixtures with a final volume of 50 μL also contained 90 mM phosphate buffer (pH 7.4), the NADP+ regenerating system (1.2 mM NADP+, 5 mM Mg2+, 5 mM isocitrate, 0.5 U/mL IDH), and 200 U/mL SOD. All incubations were performed with phosphate buffer except for the ones with CYP2A6 and CYP2C9 which were performed with 90 mM Tris buffer (pH 7.4). A positive control with 1 mg protein/mL pHLM and a blank incubation without enzyme was also performed. The reactions were started by addition of the NADP+ regenerating system and terminated by addition of 50 μL of iced-cold acetonitrile. Afterwards the mixture was centrifuged for 5 minutes at 18,407 x g and 50 μL of the supernatant were transferred to an autosampler vial. One μL was injected onto the LC−HRMS/MS system. All given concentrations are final concentrations.

2.5.2 | Monoamine oxidases activity screening

The incubations were performed at 37°C for 2 hours at a final incubation volume of 150 μL containing 25 μM of the 2C-FLY analog, MAO-A or MAO-B (0.2 mg protein/mL), 90 mM phosphate buffer, and 10 mM of the aldehyde trapping agent methoxyamine. All given concentrations were final concentrations and a negative control with MAO control was also performed. The reactions were started by addition of the enzyme and terminated with 150 μL ice-cold acetonitrile. Afterwards, the mixture was centrifuged for 2 minutes at 18,407 x g and the supernatant was transferred to an autosampler vial. Five μL were injected onto the hydrophilic interaction liquid chromatography (HILIC)−HRMS/MS system.

2.5.3 | N-Acetyltransferases activity screening

Incubations were performed at 37°C for 30 minutes with 50 μM of substrate and NAT1 or NAT2 (0.05 mg protein/mL). Besides substrate and enzyme, the incubation mixture with final volume of 150 μL also contained buffer at pH 7.5 consisting of 100 mM TEA, 500 mM ethylenediaminetetraacetic acid, and 50 mM DTT, and the CoA-system consisting of 0.1 mM AcCoA, 2.3 mM acetyl carnitine, and 0.008 U/μL AcT. First, the incubation mixture was preincubated at 37°C for 10 minutes. Subsequently, the reactions were started by addition of substrate and terminated after 30 minutes by addition of 50 μL of
<table>
<thead>
<tr>
<th>Metabolite ID</th>
<th>Metabolic Reaction</th>
<th>Characteristic ions at Measured Accurate Masses, m/z</th>
<th>Relative Intensity in MS², %</th>
<th>Calculated Exact Masses, m/z</th>
<th>Elemental Composition</th>
<th>Error, ppm</th>
<th>RT, min</th>
</tr>
</thead>
<tbody>
<tr>
<td>2C-E-FLY</td>
<td>Parent compound</td>
<td>PM at m/z 234.1486</td>
<td>8</td>
<td>234.1488</td>
<td>C₄H₂O₂N</td>
<td>-0.76</td>
<td>4.60</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Fi at m/z 217.1225</td>
<td>100</td>
<td>217.1222</td>
<td>C₄H₁O₂</td>
<td>1.24</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Fi at m/z 188.0831</td>
<td>13</td>
<td>188.0831</td>
<td>C₄H₁O₂</td>
<td>0.00</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Fi at m/z 161.0594</td>
<td>2</td>
<td>161.0596</td>
<td>C₃H₉O</td>
<td>-1.42</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Fi at m/z 133.0650</td>
<td>1</td>
<td>133.0647</td>
<td>C₃H₉O</td>
<td>2.14</td>
<td></td>
</tr>
<tr>
<td>M1</td>
<td>Hydroxylation</td>
<td>PM at m/z 250.1433</td>
<td>25</td>
<td>250.1437</td>
<td>C₄H₂O₂N</td>
<td>-1.57</td>
<td>3.36</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Fi at m/z 233.1170</td>
<td>100</td>
<td>233.1171</td>
<td>C₄H₂O₂</td>
<td>-0.62</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Fi at m/z 215.1063</td>
<td>26</td>
<td>215.1066</td>
<td>C₄H₂O₂</td>
<td>-1.30</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Fi at m/z 190.0986</td>
<td>61</td>
<td>190.0988</td>
<td>C₄H₂O₂</td>
<td>-0.81</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Fi at m/z 175.0751</td>
<td>8</td>
<td>175.0753</td>
<td>C₄H₁O₂</td>
<td>-1.03</td>
<td></td>
</tr>
<tr>
<td>M2</td>
<td>Hydroxylation</td>
<td>PM at m/z 250.1435</td>
<td>10</td>
<td>250.1437</td>
<td>C₄H₂O₂N</td>
<td>-0.77</td>
<td>3.74</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Fi at m/z 233.1171</td>
<td>64</td>
<td>233.1171</td>
<td>C₄H₂O₂</td>
<td>0.00</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Fi at m/z 215.1065</td>
<td>100</td>
<td>215.1066</td>
<td>C₄H₂O₂</td>
<td>-0.37</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Fi at m/z 203.1065</td>
<td>10</td>
<td>203.1066</td>
<td>C₄H₂O₂</td>
<td>-0.39</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Fi at m/z 187.1115</td>
<td>42</td>
<td>187.1117</td>
<td>C₃H₁O</td>
<td>-0.88</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Fi at m/z 159.0802</td>
<td>14</td>
<td>159.0804</td>
<td>C₃H₁O</td>
<td>-1.04</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Fi at m/z 131.0491</td>
<td>1</td>
<td>131.0491</td>
<td>C₃H₉O</td>
<td>0.00</td>
<td></td>
</tr>
<tr>
<td>M3</td>
<td>Dihydroxylation</td>
<td>PM at m/z 266.1392</td>
<td>32</td>
<td>266.1386</td>
<td>C₄H₂O₃N</td>
<td>2.22</td>
<td>2.13</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Fi at m/z 249.1118</td>
<td>100</td>
<td>249.1121</td>
<td>C₄H₂O₃</td>
<td>-1.04</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Fi at m/z 218.0934</td>
<td>17</td>
<td>218.0937</td>
<td>C₄H₂O₃</td>
<td>-1.23</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Fi at m/z 203.1064</td>
<td>27</td>
<td>203.1066</td>
<td>C₄H₂O₂</td>
<td>-0.88</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Fi at m/z 190.0985</td>
<td>18</td>
<td>190.0988</td>
<td>C₄H₂O₂</td>
<td>-1.34</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Fi at m/z 175.0752</td>
<td>6</td>
<td>175.0753</td>
<td>C₄H₁O₂</td>
<td>-0.45</td>
<td></td>
</tr>
<tr>
<td>M4</td>
<td>Dihydroxylation</td>
<td>PM at m/z 266.1386</td>
<td>7</td>
<td>266.1386</td>
<td>C₄H₂O₃N</td>
<td>0.00</td>
<td>2.93</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Fi at m/z 248.1276</td>
<td>24</td>
<td>248.1280</td>
<td>C₄H₁O₃N</td>
<td>-1.79</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Fi at m/z 230.1172</td>
<td>12</td>
<td>230.1175</td>
<td>C₄H₁O₃N</td>
<td>-1.21</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Fi at m/z 201.0910</td>
<td>100</td>
<td>201.0909</td>
<td>C₃H₁O₂</td>
<td>0.35</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Fi at m/z 191.1069</td>
<td>1</td>
<td>191.1066</td>
<td>C₃H₁O₂</td>
<td>1.68</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Fi at m/z 188.0830</td>
<td>5</td>
<td>188.0831</td>
<td>C₃H₁O₂</td>
<td>-0.56</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Fi at m/z 173.0959</td>
<td>5</td>
<td>173.0960</td>
<td>C₃H₁O₂</td>
<td>-0.66</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Fi at m/z 131.0490</td>
<td>1</td>
<td>131.0491</td>
<td>C₃H₁O</td>
<td>-0.76</td>
<td></td>
</tr>
<tr>
<td>M5</td>
<td>Dihydroxylation</td>
<td>PM at m/z 266.1381</td>
<td>22</td>
<td>266.1386</td>
<td>C₄H₂O₃N</td>
<td>-1.91</td>
<td>3.16</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Fi at m/z 248.1275</td>
<td>36</td>
<td>248.1280</td>
<td>C₄H₁O₃N</td>
<td>-2.19</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Fi at m/z 231.1011</td>
<td>100</td>
<td>231.1015</td>
<td>C₄H₁O₃N</td>
<td>-1.71</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Fi at m/z 213.0905</td>
<td>46</td>
<td>213.0909</td>
<td>C₃H₁O₂</td>
<td>-2.02</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Fi at m/z 203.1062</td>
<td>18</td>
<td>203.1066</td>
<td>C₃H₁O₂</td>
<td>-1.87</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Fi at m/z 185.0959</td>
<td>16</td>
<td>185.0960</td>
<td>C₃H₁O₂</td>
<td>-0.62</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Fi at m/z 157.0646</td>
<td>4</td>
<td>157.0647</td>
<td>C₃H₁O</td>
<td>-0.73</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Fi at m/z 131.0500</td>
<td>1</td>
<td>131.0491</td>
<td>C₃H₁O</td>
<td>6.87</td>
<td></td>
</tr>
<tr>
<td>M6</td>
<td>Dihydroxylation</td>
<td>PM at m/z 266.1396</td>
<td>23</td>
<td>266.1386</td>
<td>C₄H₂O₃N</td>
<td>3.73</td>
<td>3.78</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Fi at m/z 249.1119</td>
<td>23</td>
<td>249.1121</td>
<td>C₄H₁O₃N</td>
<td>-0.64</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Fi at m/z 231.1015</td>
<td>29</td>
<td>231.1015</td>
<td>C₄H₁O₃N</td>
<td>0.00</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Fi at m/z 203.1066</td>
<td>100</td>
<td>203.1066</td>
<td>C₄H₁O₃N</td>
<td>0.00</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Fi at m/z 188.0830</td>
<td>3</td>
<td>188.0831</td>
<td>C₃H₁O₂</td>
<td>-0.56</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Fi at m/z 175.0752</td>
<td>13</td>
<td>175.0753</td>
<td>C₃H₁O₂</td>
<td>-0.45</td>
<td></td>
</tr>
<tr>
<td>M7</td>
<td>Trihydroxylation</td>
<td>PM at m/z 282.1336</td>
<td>100</td>
<td>282.1335</td>
<td>C₄H₃O₄</td>
<td>0.27</td>
<td>2.72</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Fi at m/z 265.1067</td>
<td>57</td>
<td>265.1070</td>
<td>C₄H₃O₄</td>
<td>-1.03</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Fi at m/z 247.0962</td>
<td>13</td>
<td>247.0964</td>
<td>C₄H₃O₄</td>
<td>-0.85</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Fi at m/z 229.0861</td>
<td>97</td>
<td>229.0858</td>
<td>C₄H₃O₃</td>
<td>1.12</td>
<td></td>
</tr>
</tbody>
</table>

(Continues)
<table>
<thead>
<tr>
<th>Metabolite ID</th>
<th>Metabolic Reaction</th>
<th>Characteristic ions at Measured Accurate Masses, m/z</th>
<th>Relative Intensity in MS², %</th>
<th>Calculated Exact Masses, m/z</th>
<th>Elemental Composition</th>
<th>Error, ppm</th>
<th>RT, min</th>
</tr>
</thead>
<tbody>
<tr>
<td>M8</td>
<td>Carboxylation</td>
<td>PM at m/z 264.1231</td>
<td>18</td>
<td>264.1230</td>
<td>C_{14}H_{16}O_{4}N</td>
<td>0.92</td>
<td>3.43</td>
</tr>
<tr>
<td>M9</td>
<td>Hydroxylation + O-glucuronidation</td>
<td>PM at m/z 426.1757</td>
<td>16</td>
<td>426.1758</td>
<td>C_{20}H_{26}O_{9}N</td>
<td>-0.19</td>
<td>3.32</td>
</tr>
<tr>
<td>M10</td>
<td>N-acetylation</td>
<td>PM at m/z 276.1594</td>
<td>35</td>
<td>276.1593</td>
<td>C_{16}H_{22}O_{3}N</td>
<td>0.20</td>
<td>6.21</td>
</tr>
<tr>
<td>M11</td>
<td>N-acetylation + hydroxylation</td>
<td>PM at m/z 292.1542</td>
<td>92</td>
<td>292.1543</td>
<td>C_{16}H_{22}O_{4}N</td>
<td>-0.20</td>
<td>4.65</td>
</tr>
<tr>
<td>M12</td>
<td>N-acetylation + dihydroxylation</td>
<td>PM at m/z 308.1501</td>
<td>27</td>
<td>308.1492</td>
<td>C_{16}H_{22}O_{5}N</td>
<td>3.01</td>
<td>4.63</td>
</tr>
<tr>
<td>M13</td>
<td>Methoxyamine adduct</td>
<td>PM at m/z 262.1437</td>
<td>7</td>
<td>262.1437</td>
<td>C_{15}H_{20}O_{3}N</td>
<td>0.00</td>
<td>1.21</td>
</tr>
<tr>
<td>2C-EF-FLY</td>
<td>Parent compound</td>
<td>PM at m/z 252.1394</td>
<td>15</td>
<td>252.1394</td>
<td>C_{14}H_{20}O_{2}NF</td>
<td>0.00</td>
<td>4.44</td>
</tr>
<tr>
<td>M1</td>
<td>Oxidative defluorination</td>
<td>PM at m/z 250.1433</td>
<td>25</td>
<td>250.1437</td>
<td>C_{14}H_{20}O_{2}N</td>
<td>-1.57</td>
<td>3.36</td>
</tr>
<tr>
<td>M14</td>
<td>Hydroxylation</td>
<td>PM at m/z 268.1340</td>
<td>39</td>
<td>268.1343</td>
<td>C_{14}H_{20}O_{3}NF</td>
<td>-1.01</td>
<td>3.21</td>
</tr>
<tr>
<td>Metabolite ID</td>
<td>Metabolic Reaction</td>
<td>Characteristic ions at Measured Accurate Masses, m/z</td>
<td>Relative Intensity in MS², %</td>
<td>Calculated Exact Masses, m/z</td>
<td>Elemental Composition</td>
<td>Error, ppm</td>
<td>RT, min</td>
</tr>
<tr>
<td>---------------</td>
<td>-------------------</td>
<td>--------------------------------------------------</td>
<td>-----------------------------</td>
<td>-----------------------------</td>
<td>---------------------</td>
<td>-----------</td>
<td>---------</td>
</tr>
<tr>
<td>Fl at m/z 251.1074</td>
<td>100 251.1077</td>
<td>C₁₄H₁₉O₃F</td>
<td>-1.28</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fl at m/z 233.0968</td>
<td>1 233.0972</td>
<td>C₁₄H₁₀O₂</td>
<td>-1.11</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fl at m/z 213.0907</td>
<td>2 213.0909</td>
<td>C₁₂H₁₀O₂</td>
<td>-1.08</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fl at m/z 190.0985</td>
<td>18 190.0988</td>
<td>C₁₂H₁₀O₂</td>
<td>-1.34</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M15 Hydroxylation</td>
<td>PM at m/z 268.1339</td>
<td>1 268.1343</td>
<td>C₁₄H₁₀O₂NF</td>
<td>-1.39 3.38</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fl at m/z 250.1234</td>
<td>46 250.1237</td>
<td>C₁₄H₁₀O₂NF</td>
<td>-1.96</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fl at m/z 233.0968</td>
<td>1 233.0972</td>
<td>C₁₄H₁₀O₂NF</td>
<td>-1.63</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fl at m/z 213.0907</td>
<td>2 213.0909</td>
<td>C₁₂H₁₀O₂</td>
<td>-1.08</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fl at m/z 190.0985</td>
<td>18 190.0988</td>
<td>C₁₂H₁₀O₂</td>
<td>-1.34</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M16 Dihydroxylation</td>
<td>PM at m/z 284.1287</td>
<td>22 284.1292</td>
<td>C₁₄H₁₀O₂NF</td>
<td>-1.71 2.75</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fl at m/z 266.1181</td>
<td>68 266.1186</td>
<td>C₁₄H₁₀O₂NF</td>
<td>-1.50</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fl at m/z 248.0979</td>
<td>100 248.1081</td>
<td>C₁₂H₁₀O₂</td>
<td>-1.76</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fl at m/z 231.0811</td>
<td>62 231.0815</td>
<td>C₁₂H₁₀O₂</td>
<td>-1.00</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fl at m/z 217.0856</td>
<td>39 217.0858</td>
<td>C₁₂H₁₀O₂</td>
<td>-1.00</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fl at m/z 199.0753</td>
<td>29 199.0753</td>
<td>C₁₂H₁₀O₂</td>
<td>-1.00</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fl at m/z 189.0910</td>
<td>1 189.0909</td>
<td>C₁₂H₁₀O₂</td>
<td>-1.00</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M17 Dihydroxylation</td>
<td>PM at m/z 284.1290</td>
<td>48 284.1292</td>
<td>C₁₄H₁₀O₂NF</td>
<td>-1.66 3.13</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fl at m/z 266.1183</td>
<td>12 266.1186</td>
<td>C₁₄H₁₀O₂NF</td>
<td>-1.21</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fl at m/z 249.0917</td>
<td>65 249.0921</td>
<td>C₁₂H₁₀O₂</td>
<td>-1.50</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fl at m/z 231.0816</td>
<td>50 231.0815</td>
<td>C₁₂H₁₀O₂</td>
<td>-1.00</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fl at m/z 221.0970</td>
<td>100 221.0972</td>
<td>C₁₂H₁₀O₂</td>
<td>-0.71</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fl at m/z 203.0864</td>
<td>39 203.0866</td>
<td>C₁₂H₁₀O₂</td>
<td>-0.95</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M8 Oxidative defluorination + oxidation to carboxylic acid</td>
<td>PM at m/z 264.1228</td>
<td>21 264.1230</td>
<td>C₁₄H₁₀O₂N</td>
<td>-0.60 3.43</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fl at m/z 247.0966</td>
<td>100 247.0964</td>
<td>C₁₂H₁₀O₂</td>
<td>-1.07</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fl at m/z 229.0856</td>
<td>2 229.0858</td>
<td>C₁₂H₁₀O₂</td>
<td>-0.71</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fl at m/z 201.0907</td>
<td>65 201.0909</td>
<td>C₁₂H₁₀O₂</td>
<td>-1.14</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fl at m/z 173.0959</td>
<td>8 173.0960</td>
<td>C₁₂H₁₀O₂</td>
<td>-0.66</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fl at m/z 145.1010</td>
<td>2 145.1011</td>
<td>C₁₂H₁₀</td>
<td>-0.69</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M18 N-acetylation</td>
<td>PM at m/z 294.1495</td>
<td>51 294.1499</td>
<td>C₁₅H₂₁O₃NF</td>
<td>-1.43 5.79</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fl at m/z 252.1394</td>
<td>14 252.1394</td>
<td>C₁₄H₁₀O₂NF</td>
<td>-0.00</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fl at m/z 235.1128</td>
<td>100 235.1128</td>
<td>C₁₂H₁₀O₂F</td>
<td>0.00</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fl at m/z 207.0815</td>
<td>6 207.0815</td>
<td>C₁₂H₁₀O₂F</td>
<td>0.00</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fl at m/z 188.0830</td>
<td>8 188.0831</td>
<td>C₁₂H₁₀O₂</td>
<td>-0.56</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fl at m/z 159.0804</td>
<td>4 159.0804</td>
<td>C₁₂H₁₀O</td>
<td>0.00</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M19 Methoxyamine adduct</td>
<td>PM at m/z 280.1336</td>
<td>9 280.1343</td>
<td>C₁₅H₂₁O₃NF</td>
<td>-0.61 1.23</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fl at m/z 221.0972</td>
<td>100 221.0972</td>
<td>C₁₅H₂₁O₂F</td>
<td>0.00</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fl at m/z 208.0894</td>
<td>19 208.0893</td>
<td>C₁₅H₂₁O₂F</td>
<td>0.32</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fl at m/z 188.0831</td>
<td>1 188.0831</td>
<td>C₁₅H₂₁O₂</td>
<td>0.00</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2C-T-7-FLY Parent compound</td>
<td>PM at m/z 280.1359</td>
<td>10 280.1365</td>
<td>C₁₅H₂₁O₂NS</td>
<td>-2.14 5.29</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fl at m/z 263.1095</td>
<td>100 263.1099</td>
<td>C₁₅H₂₁O₂S</td>
<td>-1.71</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fl at m/z 221.0627</td>
<td>26 221.0630</td>
<td>C₁₂H₁₀O₂S</td>
<td>-1.36</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fl at m/z 188.0827</td>
<td>12 188.0831</td>
<td>C₁₂H₁₀O₂S</td>
<td>-1.36</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fl at m/z 159.0801</td>
<td>2 159.0804</td>
<td>C₁₂H₁₀O₂</td>
<td>-1.67</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fl at m/z 131.0854</td>
<td>1 131.0855</td>
<td>C₁₂H₁₀</td>
<td>-0.38</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M20 Oxidation to sulfoxide</td>
<td>PM at m/z 296.1309</td>
<td>88 296.1314</td>
<td>C₁₅H₂₁O₂NS</td>
<td>-1.06 3.80</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fl at m/z 279.1049</td>
<td>14 279.1049</td>
<td>C₁₂H₁₀O₂S</td>
<td>-2.74</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fl at m/z 237.0575</td>
<td>100 237.0579</td>
<td>C₁₂H₁₀O₂S</td>
<td>-1.33</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

(Continues)
<table>
<thead>
<tr>
<th>Metabolite ID</th>
<th>Metabolic Reaction</th>
<th>Characteristic ions at Measured Accurate Masses, m/z</th>
<th>Relative Intensity in MS², %</th>
<th>Calculated Exact Masses, m/z</th>
<th>Elemental Composition</th>
<th>Error, ppm</th>
<th>RT, min</th>
</tr>
</thead>
<tbody>
<tr>
<td>M21 Hydroxylation PM at m/z 296.1310</td>
<td>4</td>
<td>296.1314</td>
<td>C₁₁H₂₀O₃NS</td>
<td>-1.40</td>
<td>3.98</td>
<td></td>
<td></td>
</tr>
<tr>
<td>M22 Oxidation to sulfone PM at m/z 312.1262</td>
<td>70</td>
<td>312.1263</td>
<td>C₁₅H₂₂O₄NS</td>
<td>-0.73</td>
<td>2.83</td>
<td></td>
<td></td>
</tr>
<tr>
<td>M23 Dihydroxylation PM at m/z 312.1261</td>
<td>63</td>
<td>312.1263</td>
<td>C₁₅H₂₀O₄NS</td>
<td>-0.73</td>
<td>3.91</td>
<td></td>
<td></td>
</tr>
<tr>
<td>M24 Carboxylation PM at m/z 310.1104</td>
<td>42</td>
<td>310.1107</td>
<td>C₁₅H₂₀O₄NS</td>
<td>-0.90</td>
<td>4.00</td>
<td></td>
<td></td>
</tr>
<tr>
<td>M25 S-Dealkylation PM at m/z 238.0889</td>
<td>1</td>
<td>238.0895</td>
<td>C₁₂H₁₆O₂NS</td>
<td>-2.72</td>
<td>4.17</td>
<td></td>
<td></td>
</tr>
<tr>
<td>M26 N-acetylation PM at m/z 322.1463</td>
<td>42</td>
<td>322.1471</td>
<td>C₁₇H₂₄O₅NS</td>
<td>-2.37</td>
<td>6.85</td>
<td></td>
<td></td>
</tr>
<tr>
<td>M27 N-acetylation + oxidation to sulfoxide PM at m/z 338.1411</td>
<td>100</td>
<td>338.1420</td>
<td>C₁₇H₂₄O₅NS</td>
<td>-2.60</td>
<td>5.21</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

(Continues)
ice-cold acetonitrile. The mixture was centrifuged for 5 minutes at 18,007 x g and 50 μL of the supernatant was transferred to an autosampler vial. One μL was injected onto the LC–HRMS/MS system.

### 2.6 LC–HRMS/MS conditions

Analyses were performed using a Thermo Fisher scientific (TF, Dreieich, Germany) Dionex UltiMate 3000 Rapid Separation (RS) UHPLC system with a quaternary UltiMate 3000 RS pump and an UltiMate 3000 RS autosampler controlled by the TF Chromeleon software version 6.80. It was coupled to a TF Q-Exactive Plus, equipped with a heated electrospray ionization II source (HESI II). The mass spectrometer was mass calibrated before analysis using a Positive Mode Cal Mix (Supelco, Bellefonte, PA, USA).

For all measurements except the MAO activity screening, the LC conditions were as follows: TF Accucore Phenyl/Hexyl column (100 mm x 2.1 mm inside diameter, ID, 2.6 μm particle size); gradient elution with eluent A (2mM aqueous ammonium formate solution containing 0.1% (v/v) formic acid) and eluent B (ammonium formate solution with acetonitrile/methanol (50:50, v/v) containing 0.1% (v/v) formic acid and 1% (v/v) water). The gradient was set as follows: 0–1 minute 1% B, 1–10 minutes to 99% B, 10–11.5 minutes hold 99% B, 11.5–13.5 minutes hold 1% B. The flow rate settings were as follows: 0–11.5 minutes at 0.500 mL/min, and 11.5–13.5 minutes at 0.800 mL/min.

Conditions for the MAO activity screenings were as follows: Macherey-Nagel (Düren, Germany) HIUC Nucleodur column (125 mm x 3 mm ID, 3 μm particle size). As mobile phases eluent C (200mM aqueous ammonium acetate) and eluent D (acetonitrile containing 0.1%, v/v, formic acid) were used. The gradient was set as follows: 0–8.5 minutes 98% D to 40% D, 8.5–10 minutes hold 40% D, 10–12 min hold 98% D. The flow rate was set to 0.500 mL/min.

The HESI-II conditions were as follows: Heater temperature, 320°C; ion transfer capillary temperature, 320°C; sheath gas, 60 arbitrary units (AU); auxiliary gas, 10 AU; spray voltage, 4.00 kV, positive mode; S-lens RF level, 50.0. Mass spectrometry was performed in positive electrospray ionization (ESI) full-scan mode and targeted MS² mode with an inclusion list containing masses of expected metabolites. The settings for full-scan data acquisition were chosen as follows: resolution, 35 000; automatic gain control (AGC) target, 1e6; maximum injection time (IT), 120 ms; scan range, m/z 200–1200 (2C-E-FLY), m/z 200–1100 (2C-FLY), or m/z 200–800 (2C-T-FLY). The settings for the targeted MS² mode were chosen as follows: resolution, 17,500; AGC target, 2e5; maximum IT, 250 ms; isolation window, m/z 1.0; stepped normalized collision energy (NCE) with steps 17.5%, 35%, 52.5%; pick others, enabled. For data evaluation, TF Xcalibur Qual Browser software version 2.2 SP 1.48 was used.
2.7 Toxicological detectability in rat urine

Standard urine screening approaches (SUSAs) by gas chromatography (GC)-MS, LC-ion trap (IT)-MS, and LC-HRMS/MS were performed as described before.16,18,19,24 Briefly, urine precipitation with acetonitrile was performed for the LC-based SUSAs and liquid–liquid extraction (LLE) after acidic hydrolysis followed by acetylation for the GC-MS SUSA.

3 RESULTS AND DISCUSSION

3.1 Identification of metabolites

For identification of metabolites, MS\textsuperscript{1} data after analysis of the in vivo and in vitro samples were screened for exact precursor masses (PMs) of expected metabolites. A maximum deviation of 5 ppm between measured and calculated exact PM was accepted. Afterwards, the

<table>
<thead>
<tr>
<th>Metabolite iD</th>
<th>Metabolic Reaction</th>
<th>Calculated Exact Masses, m/z</th>
<th>Rat (UP)</th>
<th>SPE</th>
<th>1 h</th>
<th>6 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>2C-E-FLY</td>
<td>Parent compound</td>
<td>234.1488</td>
<td>2.35E+07</td>
<td>5.23E+08</td>
<td>1.11E+09</td>
<td>1.27E+09</td>
</tr>
<tr>
<td>M1</td>
<td>Hydroxylation</td>
<td>250.1437</td>
<td>1.23E+08</td>
<td>1.39E+09</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>M2</td>
<td>Hydroxylation</td>
<td>250.1437</td>
<td>2.19E+08</td>
<td>3.17E+08</td>
<td>9.14E+05</td>
<td>1.87E+06</td>
</tr>
<tr>
<td>M3</td>
<td>Dihydroxylation</td>
<td>266.1386</td>
<td>4.45E+06</td>
<td>4.84E+07</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>M4</td>
<td>Dihydroxylation</td>
<td>266.1386</td>
<td>5.51E+07</td>
<td>4.59E+07</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>M5</td>
<td>Dihydroxylation</td>
<td>266.1386</td>
<td>5.61E+07</td>
<td>2.12E+08</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>M6</td>
<td>Dihydroxylation</td>
<td>266.1386</td>
<td>1.19E+06</td>
<td>1.97E+07</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>M7</td>
<td>Trihydroxylation</td>
<td>282.1335</td>
<td>9.13E+06</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>M8</td>
<td>Carboxylation</td>
<td>264.1230</td>
<td>1.32E+07</td>
<td>1.30E+08</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>M9</td>
<td>Hydroxylation + O-glucuronidation</td>
<td>426.1758</td>
<td>7.09E+06</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>M10</td>
<td>N-acetylation</td>
<td>276.1593</td>
<td>1.91E+07</td>
<td>7.17E+05</td>
<td>1.52E+07</td>
<td>8.40E+07</td>
</tr>
<tr>
<td>M11</td>
<td>N-acetylation + hydroxylation</td>
<td>292.1543</td>
<td>6.20E+07</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>M12</td>
<td>N-acetylation + dihydroxylation</td>
<td>308.1492</td>
<td>4.37E+06</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2C-EF-FLY</td>
<td>Parent compound</td>
<td>252.1394</td>
<td>7.62E+07</td>
<td>1.00E+09</td>
<td>6.23E+08</td>
<td>1.38E+09</td>
</tr>
<tr>
<td>M1</td>
<td>Oxidative defluorination</td>
<td>250.1437</td>
<td>1.65E+07</td>
<td>6.68E+07</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>M14</td>
<td>Hydroxylation</td>
<td>268.1343</td>
<td>-</td>
<td>1.11E+08</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>M15</td>
<td>Hydroxylation</td>
<td>268.1343</td>
<td>1.78E+08</td>
<td>5.41E+08</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>M16</td>
<td>Dihydroxylation</td>
<td>284.1292</td>
<td>2.72E+07</td>
<td>6.39E+07</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>M17</td>
<td>Dihydroxylation</td>
<td>284.1292</td>
<td>5.93E+06</td>
<td>2.32E+07</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>M8</td>
<td>Oxidative defluorination + oxidation to carboxylic acid</td>
<td>264.1230</td>
<td>1.54E+07</td>
<td>6.11E+07</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>M18</td>
<td>N-acetylation</td>
<td>294.1499</td>
<td>1.03E+07</td>
<td>7.00E+05</td>
<td>3.52E+06</td>
<td>4.29E+07</td>
</tr>
<tr>
<td>2C-T-7-FLY</td>
<td>Parent compound</td>
<td>280.1365</td>
<td>5.19E+07</td>
<td>5.23E+08</td>
<td>5.54E+08</td>
<td>5.15E+08</td>
</tr>
<tr>
<td>M20</td>
<td>Oxidation to sulfide</td>
<td>296.1314</td>
<td>3.12E+07</td>
<td>2.98E+08</td>
<td>7.16E+05</td>
<td>1.96E+06</td>
</tr>
<tr>
<td>M21</td>
<td>Hydroxylation</td>
<td>296.1314</td>
<td>6.24E+07</td>
<td>7.12E+08</td>
<td>7.37E+05</td>
<td>2.11E+06</td>
</tr>
<tr>
<td>M22</td>
<td>Oxidation to sulfone</td>
<td>312.1263</td>
<td>7.46E+06</td>
<td>8.29E+07</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>M23</td>
<td>Dihydroxylation</td>
<td>312.1263</td>
<td>-</td>
<td>1.41E+07</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>M24</td>
<td>Carboxylation</td>
<td>310.1107</td>
<td>2.63E+07</td>
<td>1.98E+08</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>M25</td>
<td>S-Dealkylation</td>
<td>238.0895</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>M26</td>
<td>N-acetylation</td>
<td>322.1471</td>
<td>8.59E+05</td>
<td>1.19E+07</td>
<td>4.01E+07</td>
<td></td>
</tr>
<tr>
<td>M27</td>
<td>N-acetylation + oxidation to sulfide</td>
<td>338.1420</td>
<td>1.71E+08</td>
<td>1.68E+07</td>
<td>9.08E+04</td>
<td>2.28E+06</td>
</tr>
<tr>
<td>M28</td>
<td>N-acetylation + oxidation to sulfone</td>
<td>354.1369</td>
<td>1.31E+09</td>
<td>7.29E+07</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>M29</td>
<td>N-acetylation + carboxylation</td>
<td>352.1212</td>
<td>2.58E+08</td>
<td>1.11E+07</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
Fragmentation patterns in their MS² spectra were interpreted and compared to those of the parent compounds. Analytical information of the parent compounds and all phase I and II metabolites are listed in Tables 1 and 2. The given masses are the calculated exact masses. In total, the analyses of rat urine and in vitro incubations resulted in detections of several tentative metabolites: 2C-E-FLY, 13 metabolites; 2C-EF-FLY, 8 metabolites; and 2C-T-7-FLY, 11 metabolites. The resulting metabolic pathways can be found in Figures 2–4.

Fragmentation patterns of 2C-E-FLY and its metabolites are discussed exemplarily in the following as data of 2C-EF-FLY, 2C-T-7-FLY, and their metabolites were comparable. The MS² spectrum of the parent compound 2C-E-FLY (M + H⁺, PM at m/z 234.1488, C₁₄H₂₀O₂N) showed an initial loss of ammonia (−17 u, NH₃, fragment ion, FI at m/z 217.1222, C₁₄H₁₇O₂). Afterwards, an ethyl moiety was eliminated (−29 u, C₂H₅, FI at m/z 188.0831, C₁₃H₁₅O₂). The FI at m/z 161.0596 (C₁₀H₇O₂) represented the intact 2,3,6,7-tetrahydrobenzo[1,2-b:4,5-b']difuran core before cleavage of one of the tetrahydrofuran rings with only a methyl group remaining at the benzene ring (FI at m/z 133.0647, C₉H₇O). The hydroxy metabolites (M1 and 2) also showed the loss of ammonia (FI at m/z 233.1171, C₁₄H₁₇O₃) and afterwards an elimination of water (−18 u, H₂O) resulting in a shift of 2 u from FI at m/z 217.1222 (2C-E-FLY) to 215.1066 (M1 and 2, C₁₄H₁₅O₂). For M1, the position of the hydroxy group was considered outside the 2,3,6,7-tetrahydrobenzo[1,2-b:4,5-b']difuran core. The double bond at the ethyl moiety formed after loss of water was afterwards eliminated resulting in the FI at m/z 190.0988 (C₁₂H₁₄O₂) and 175.0753 (C₁₁H₁₄O₂) representing an unchanged 2,3,6,7-tetrahydrobenzo[1,2-b:4,5-b']difuran core with a remaining ethyl and methyl moiety, respectively. It is likely, that the hydroxy group was located at the terminal carbon atom of the ethyl moiety because the corresponding carboxy metabolite (M8) was also identified. In contrast to M1, M2 was probably hydroxylated at the 2,3,6,7-tetrahydrobenzo[1,2-b:4,5-b']difuran core due to the FI at m/z 131.0491 (C₉H₇O), which had an additional double bond in comparison to the FI at m/z 133.0647 (2C-E-FLY). The FI at m/z 215.1066 (C₁₄H₁₅O₂), 187.1117 (C₁₃H₁₅O), and 159.0804 (C₁₁H₁₄O₂) also provided a double bond inside of the ring system. However, exact positions of the hydroxy groups could not be identified based on fragmentation patterns.

Four dihydroxy metabolites (M3–M6) could be identified. For M3, the hydroxy groups were located at the ethyl moiety and the ethyl amine part as confirmed by the FI 175.0753 (C₁₁H₁₄O₂) with an unchanged 2,3,6,7-tetrahydrobenzo[1,2-b:4,5-b']difuran core. M4 and M5 most probably had one of their hydroxy groups at the 2,3,6,7-tetrahydrobenzo[1,2-b:4,5-b']difuran core due to the FI at m/z 131.0491 in accordance to M2. M5 was thought to also contain a hydroxy group at the ethyl moiety and represented therefore a combination of M1 and M2. In the case of M6, both hydroxylations are

![Figure 2](image_url)
determined to be located at the ethyl moiety. Again, initial steps were loss of ammonia and water, followed by the elimination of CO ($-28$ u) from the FI at m/z 231.1015 (C$_{14}$H$_{15}$O$_3$) leading to the FI at m/z 203.1066 (C$_{13}$H$_{15}$O$_2$). Furthermore, the presence of FI at m/z 175.0753 (C$_{11}$H$_{11}$O$_2$) indicated an unchanged 2,3,6,7-tetrahydrobenzo[1,2-b:4,5-b’]difuran core. One trihydroxy metabolite (M7) could also be identified. The absence of a hydroxy group at the 2,3,6,7-tetrahydrobenzo[1,2-b:4,5-b’]difuran core was confirmed by the FI at m/z 175.0753 (C$_{11}$H$_{11}$O$_2$). According to Niessen et al, the carboxylic acid, such as that found in the carboxy metabolite (M8), can be eliminated in two steps.25 After initial loss of the ammonia (FI at m/z 247.0964, C$_{14}$H$_{15}$O$_4$), the elimination of water ($-18$ u, H$_2$O) led to the FI at m/z 229.0858 (C$_{14}$H$_{13}$O$_3$) followed by a rearrangement of the double bonds resulting in the formation of an oxonium ion. Subsequently, CO was eliminated and led to the FI at m/z 201.0909 (C$_{13}$H$_{13}$O$_2$). It should be considered, that M1 and M8 can also be formed during metabolic transformation of 2C-EFLY after oxidative defluorination. In conclusion, both metabolites are not suitable for unambiguous identification of an intake of 2C-EFLY or 2C-EFLY and could lead to an incorrect interpretation of analytical findings.

Concerning phase II metabolites of 2C-EFLY, one O-glucuronide and three N-acetylated metabolites could be detected (M9–M12). The fragmentation pattern of the O-glucuronide (M9, PM at m/z 426.1758, C$_{20}$H$_{28}$O$_9$N) was in accordance to the corresponding phase I metabolite (M2) after elimination of glucuronic acid ($-176$ u, C$_{6}$H$_{8}$O$_6$). After elimination of the acetyl moiety ($-42$ u, C$_{2}$H$_{2}$O), the N-acetyl metabolite (M10, PM at m/z 276.1593, C$_{16}$H$_{22}$O$_3$N) provided the same fragmentation pattern as 2C-EFLY. M2 and M5 were also found to be N-acetylated in vivo (M11 + 12). In comparison to the corresponding phase I metabolites, M11 and M12 provided additional FI in their MS$^2$ spectrum (M11 at m/z 274.1437, C$_{16}$H$_{20}$O$_3$N, and M12 at m/z 290.1386, C$_{14}$H$_{20}$O$_4$N, and m/z 272.1280, C$_{14}$H$_{20}$O$_3$N) formed after elimination of water ($-18$ u, H$_2$O) before elimination of the acetyl moiety.

In summary, most metabolites were detected in rat urine after precipitation and only few metabolites were formed in incubations with pS9 fraction. This was most probably caused by the different experimental conditions. First of all, pS9 incubations were stopped after a maximum of 6 hours, while rat urine was collected over a 24-hour period. In addition, in vitro experiments have limitations concerning distribution and excretion leading to simple metabolites formed after few reaction steps. Last but not least, species differences can be followed by formation of different metabolites. However, to conclude which model is most suitable for developing urine screening approaches, authentic human urine would be needed. An intake by humans in the framework of a controlled trial would be the gold standard, but is...
FIGURE 4  Metabolic pathways of 2C-T-7-FLY detected in rat urine or in in vitro incubations with pooled human liver S9 fraction (pS9), pooled human liver microsomes (pHLMs), or recombinant human monoamine oxidases (MAOs)

TABLE 3  General involvement of tested cytochrome P450 (CYP) isoenzymes and flavin-containing monooxygenase 3 (FMO3) in metabolic phase I steps. -, not detected

<table>
<thead>
<tr>
<th>Metabolic step</th>
<th>2C-E-FLY</th>
<th>2C-EF-FLY</th>
<th>2C-T-7-FLY</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydroxylation (chain)</td>
<td>CYP2D6 CYP3A4</td>
<td>CYP2D6</td>
<td>CYP2D6</td>
</tr>
<tr>
<td>Hydroxylation (core)</td>
<td>CYP2D6</td>
<td>CYP2D6</td>
<td>-</td>
</tr>
<tr>
<td>Oxidation to sulfoxide</td>
<td>-</td>
<td>-</td>
<td>CYP3A4</td>
</tr>
<tr>
<td>S-Dealkylation</td>
<td>-</td>
<td>-</td>
<td>CYP2D6 CYP3A4</td>
</tr>
</tbody>
</table>

TABLE 4  Toxicological detectability of 2C-E-FLY, 2C-EF-FLY, and 2C-T-7-FLY in rat urine by the GC–MS standard urine screening approach. BW, body weight; +, detected; -, not detected; AC, acetylated

<table>
<thead>
<tr>
<th>Compound</th>
<th>Precursor Ion Mass, m/z</th>
<th>Elemental Composition</th>
<th>Retention Index (RI)</th>
<th>Characteristic Fragment Ions, m/z</th>
<th>Administered Dose, mg/kg BW</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.2</td>
</tr>
<tr>
<td>2C-E-FLY-M (hydroxy) -H2O</td>
<td>273</td>
<td>C_{16}H_{19}O_{3}N</td>
<td>2320</td>
<td>199, 214</td>
<td>-</td>
</tr>
<tr>
<td>2C-EF-FLY AC</td>
<td>293</td>
<td>C_{16}H_{20}O_{3}NF</td>
<td>2480</td>
<td>221, 234</td>
<td>+</td>
</tr>
<tr>
<td>2C-EF-FLY-M (hydroxy) -H2O</td>
<td>291</td>
<td>C_{16}H_{19}O_{3}NF</td>
<td>2450</td>
<td>199, 219, 232</td>
<td>+</td>
</tr>
<tr>
<td>2C-EF-FLY-M (dihydroxy) -2H2O AC</td>
<td>289</td>
<td>C_{16}H_{18}O_{3}NF</td>
<td>2420</td>
<td>197, 217, 230</td>
<td>+</td>
</tr>
<tr>
<td>2C-T-7-FLY AC</td>
<td>321</td>
<td>C_{17}H_{23}O_{3}NS</td>
<td>2720</td>
<td>207, 219, 249, 262</td>
<td>-</td>
</tr>
<tr>
<td>2C-T-7-FLY-M (hydroxy) -H2O</td>
<td>319</td>
<td>C_{17}H_{22}O_{3}NS</td>
<td>2690</td>
<td>205, 247, 260</td>
<td>-</td>
</tr>
<tr>
<td>2C-T-7-FLY-M (hydroxy) 2 AC</td>
<td>379</td>
<td>C_{19}H_{25}O_{3}NS</td>
<td>2890</td>
<td>101, 218, 260, 320</td>
<td>-</td>
</tr>
<tr>
<td>2C-T-7-FLY-M (dihydroxy) -H2O 2 AC</td>
<td>377</td>
<td>C_{19}H_{23}O_{3}NS</td>
<td>2850</td>
<td>101, 216, 258, 318</td>
<td>-</td>
</tr>
</tbody>
</table>
considered as unethical, time consuming, and expensive.\textsuperscript{26} Nevertheless, human biosamples after intake of NPS derived from authentic cases are sometimes available and should then be used for a comparative study summarizing all metabolites detected in vitro and in vivo.

3.2 | Investigation of isoenzyme activities

3.2.1 | Monoxygenases activity screening

The monoxygenases activity screening was performed to investigate the impact of ten CYP isoenzymes and FMO3 on phase I metabolism of the three 2C-FLY derivatives. Incubations with pHLM were used as positive control. Results are summarized in Table 3. The side chain hydroxylation of 2C-EF-FLY and 2C-T-7-FLY was catalyzed by CYP2D6 and additionally by CYP3A4 in case of 2C-E-FLY. Hydroxylation of the 2,3,6,7-tetrahydrobenzo[1,2-b:4,5-b]difuran core of 2C-E-FLY and 2C-FLY was catalyzed by CYP2D6. The thioether moiety of 2C-T-7-FLY was oxidized to the sulfoxide (M20) by CYP3A4 and FMO3. Side chain dealkylation of 2C-T-7-FLY (M25) was catalyzed by CYP2D6 and CYP3A4 and was detected neither in rat urine nor in pS9 incubations.

3.2.2 | Monoamine oxidases activity screening

Due to the primary amine contained in the structure of the 2C-FLY drugs, a deamination catalyzed by MAO isoforms was likely to occur and this metabolic step was already described for 2C compounds.\textsuperscript{14,27} However, due to the loss of nitrogen, neither the detection of the deaminated metabolite nor the expected end-products (oxidized carboxylic acid or reduced alcohol) was possible with the used settings. Therefore, incubations with MAO-A or B and the aldehyde trapping agent methoxyamine were performed. The deaminated metabolites (M13, 19, 30) could afterwards be detected as oxime adducts of the 2C-FLY drugs and methoxyamine. For M13 (PM at m/z 262.1437, C\textsubscript{15}H\textsubscript{12}O\textsubscript{3}N), the oxime moiety plus one carbon atom was eliminated resulting in FI at m/z 203.1066 (C\textsubscript{13}H\textsubscript{15}O\textsubscript{2}) in the first fragmentation step. Afterwards, the ethyl moiety was eliminated in subsequent steps (FI at m/z 190.0988, C\textsubscript{12}H\textsubscript{14}O\textsubscript{2}, and 175.0753, C\textsubscript{11}H\textsubscript{12}O\textsubscript{2}). The deamination of all three 2C-FLY derivatives was found to be catalyzed by MAO-A and B. Further kinetic studies would allow to assess, which isoenzyme is the main catalyzing isomerase.

3.2.3 | N-Acetyltransferases activity screening

N-Acetylation of the primary amine was found to be one of the main metabolic steps. In order to investigate which NAT isoenzyme catalyzed this reaction, a NAT activity screening was performed and both isoforms, NAT1 and NAT2, catalyzed the reaction. Again, further kinetic studies would allow assessment of which isoenzyme is the main catalyzing isomerase.

3.3 | Toxicological detectability in rat urine

No information concerning dosage of 2C-E-FLY, 2C-EF-FLY, and 2C-T-7-FLY in humans could be found but oral 2C-B-FLY doses of 10–20 mg were described.\textsuperscript{34} This was comparable to 2C-B dosages of 12–24 mg as recommended by Shulgin and Shulgin, who also listed dosages of 10–30 mg for 2C-E and 2C-T-7.\textsuperscript{28} Rat doses of 0.2 (low dose) or 2 mg/kg BW (high dose) corresponded to human doses of 0.03 and 0.33 mg/kg BW, respectively,\textsuperscript{29} which would be a human oral dose of 2 or 23 mg for a BW of 70 kg. Therefore, recreational doses should be

<table>
<thead>
<tr>
<th>Metabolite iD</th>
<th>Parent Compound or Metabolite</th>
<th>Precursor Ion Mass, m/z</th>
<th>Retention Time, Min</th>
<th>Characteristic MS\textsuperscript{2} Fragment Ions at m/z</th>
<th>Characteristic MS\textsuperscript{3} Fragment Ions at m/z</th>
<th>Administered Dose, mg/kg BW</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parent compound</td>
<td>2C-E-FLY</td>
<td>234</td>
<td>10.38</td>
<td>217, 189, 188, 171, 161</td>
<td>(189) 161, 133</td>
<td>0.2</td>
</tr>
<tr>
<td>M1</td>
<td>2C-E-FLY-M (hydroxy)</td>
<td>250</td>
<td>5.94</td>
<td>215, 190, 189, 162</td>
<td>(190) 162, 147, 133</td>
<td>2</td>
</tr>
<tr>
<td>M2</td>
<td>2C-E-FLY-M (hydroxy)</td>
<td>250</td>
<td>6.70</td>
<td>215, 203, 187, 159</td>
<td>(215) 187, 159</td>
<td>2</td>
</tr>
<tr>
<td>M4</td>
<td>2C-E-FLY-M (dihydroxy)</td>
<td>266</td>
<td>4.90</td>
<td>231, 230, 219, 201</td>
<td>(230) 201</td>
<td>2</td>
</tr>
<tr>
<td>M5</td>
<td>2C-E-FLY-M (dihydroxy)</td>
<td>266</td>
<td>5.76</td>
<td>231, 230, 213</td>
<td>(230) 202, 185, 147</td>
<td>2</td>
</tr>
<tr>
<td>M10</td>
<td>2C-E-FLY-M (N-acetyl)</td>
<td>276</td>
<td>15.32</td>
<td>240, 234, 220, 176, 142, 129</td>
<td>(220) 142, 129</td>
<td>2</td>
</tr>
<tr>
<td>M8</td>
<td>2C-EF-FLY-M (oxidative defluorination and oxidation)</td>
<td>264</td>
<td>7.45</td>
<td>229, 201, 187, 159, 109</td>
<td>(229) 201, 187</td>
<td>2</td>
</tr>
<tr>
<td>M21</td>
<td>2C-T-7-FLY-M (hydroxy)</td>
<td>296</td>
<td>7.57</td>
<td>261, 221, 189, 161, 149</td>
<td>(189) 161, 149</td>
<td>2</td>
</tr>
<tr>
<td>M28</td>
<td>2C-T-7-FLY (N-acetyl sulfone)</td>
<td>354</td>
<td>6.81</td>
<td>294, 247, 235, 188</td>
<td>(247) 188</td>
<td>2</td>
</tr>
</tbody>
</table>

TABLE 5 | Toxicological detectability of 2C-E-FLY, 2C-EF-FLY, and 2C-T-7-FLY in rat urine by the LC–IT–MS standard urine screening approach. Precursor ions of the MS\textsuperscript{3} fragment ions are given in brackets. Metabolite iDs correspond to Table 1. BW, body weight; D, precursor ion in MS\textsuperscript{1} detected; I, identified due to MS\textsuperscript{2} spectrum.
expected to be in the range of the high dose, whereas doses resulting in severe intoxications are expected to be even higher.

### 3.3.3 | LC–HRMS/MS SUSA

Results are summarized in Table 6. Similar to the LC–IT–MS SUSA, the three 2C-E-FLY drugs and/or their metabolites could be identified in rat urine after low and high dose administration by the corresponding MS\(^2\) spectra. An intake is thus expected to be detectable by the LC–HRMS/MS SUSA.

### 4 | CONCLUSIONS

In total, 32 metabolites of 2C-E-FLY, 2C-EF-FLY, and 2C-T-7-FLY could be identified in rat urine and in vitro incubations. Hydroxylation and N-acetylation were identified as main metabolic steps, whereas methoxamine was successfully used for detection of the deaminated metabolites formed by MAO-A and B. Most metabolites were identified in rat urine after precipitation. Phase I metabolic reactions were mainly catalyzed by CYP2D6 and CYP3A4 and N-acetylation by NAT1 and NAT2. Intoxications with these NPS should be detectable by all three tested SUSAs, but common metabolites of 2C-E-FLY (M1, M8) have to be considered during interpretation of analytical findings.

### ACKNOWLEDGEMENTS

The authors would like to thank Gabriele Ulrich, Hans H. Maurer, and Armin A. Weber for their support.

---

**TABLE 6** Toxicological detectability of 2C-E-FLY, 2C-EF-FLY, and 2C-T-7-FLY in rat urine by the LC-HRMS/MS standard urine screening approach. Metabolite IDs correspond to Table 1. BW, body weight; -, not detected; D, precursor ion in MS\(^1\) detected; I, identified due to MS\(^2\) spectrum.

<table>
<thead>
<tr>
<th>Metabolite ID</th>
<th>Parent Compound or Metabolite</th>
<th>Exact Precursor Ion Mass, m/z</th>
<th>Retention Time, Min</th>
<th>Administered Dose, mg/kg BW</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parent compound</td>
<td>2C-E-FLY</td>
<td>234.1488</td>
<td>6.20</td>
<td>D</td>
</tr>
<tr>
<td>M1</td>
<td>2C-E-FLY-M (hydroxy)</td>
<td>250.1437</td>
<td>4.95</td>
<td>D</td>
</tr>
<tr>
<td>M2</td>
<td>2C-E-FLY-M (hydroxy)</td>
<td>250.1437</td>
<td>5.29</td>
<td>I</td>
</tr>
<tr>
<td>M4</td>
<td>2C-E-FLY-M (dihydroxy)</td>
<td>266.1386</td>
<td>4.50</td>
<td>D</td>
</tr>
<tr>
<td>M5</td>
<td>2C-E-FLY-M (dihydroxy)</td>
<td>266.1386</td>
<td>4.77</td>
<td>D</td>
</tr>
<tr>
<td>M10</td>
<td>2C-E-FLY-M (N-acetyl)</td>
<td>276.1593</td>
<td>7.77</td>
<td>D</td>
</tr>
<tr>
<td>M11</td>
<td>2C-E-FLY-M (N-acetyl hydroxy)</td>
<td>292.1543</td>
<td>6.14</td>
<td>D</td>
</tr>
<tr>
<td>Parent compound</td>
<td>2C-EF-FLY</td>
<td>252.1394</td>
<td>5.80</td>
<td>I</td>
</tr>
<tr>
<td>M15</td>
<td>2C-EF-FLY-M (hydroxy)</td>
<td>268.1343</td>
<td>4.99</td>
<td>I</td>
</tr>
<tr>
<td>M8</td>
<td>2C-EF-FLY-M (carboxy)</td>
<td>264.1230</td>
<td>5.02</td>
<td>I</td>
</tr>
<tr>
<td>M18</td>
<td>2C-EF-FLY-M (N-acetyl)</td>
<td>294.1499</td>
<td>7.40</td>
<td>I</td>
</tr>
<tr>
<td>Parent compound</td>
<td>2C-T-7-FLY</td>
<td>280.1365</td>
<td>6.88</td>
<td>I</td>
</tr>
<tr>
<td>M20</td>
<td>2C-T-7-FLY-M (sulfoxide)</td>
<td>296.1314</td>
<td>5.37</td>
<td>I</td>
</tr>
<tr>
<td>M21</td>
<td>2C-T-7-FLY-M (hydroxy)</td>
<td>296.1314</td>
<td>5.55</td>
<td>I</td>
</tr>
<tr>
<td>M24</td>
<td>2C-T-7-FLY-M (carboxy)</td>
<td>310.1107</td>
<td>5.70</td>
<td>I</td>
</tr>
<tr>
<td>M27</td>
<td>2C-T-7-FLY-M (N-acetyl sulfoxide)</td>
<td>338.1420</td>
<td>6.69</td>
<td>I</td>
</tr>
<tr>
<td>M28</td>
<td>2C-T-7-FLY-M (N-acetyl dihydroxy)</td>
<td>354.1369</td>
<td>5.65</td>
<td>I</td>
</tr>
<tr>
<td>M29</td>
<td>2C-T-7-FLY-M (N-acetyl carboxy)</td>
<td>352.1213</td>
<td>6.89</td>
<td>I</td>
</tr>
</tbody>
</table>
CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

ORCID

Simon D. Brandt  https://orcid.org/0000-0001-8632-5372
Markus R. Meyer  https://orcid.org/0000-0003-4377-6784

REFERENCES


