SUPPLEMENTARY INFORMATION

Structural elucidation of major selective androgen receptor modulator (SARM) metabolites for doping control

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Supporting Figures

Figure S1: $^{19}$F NMR spectrum of purified sulphated metabolite 5.

Figure S2: Retention time comparison of the urinary natural and the synthesized purified cyano SARMs-derived metabolite 5. Extracted ion chromatograms on a C18 column for the urinary, synthesized and mixed samples (top right corner of each chromatogram: signal intensities).
Figure S3: Synthesis of purified sulphated metabolite 7. (i) NIS, MeOH/THF, 91%; (ii) CuI, Cs₂CO₃, 1,10-phenanthroline mono hydrate, MeOH, 29%; (iii) BBr₃, DCM, 40%; (iv) SO₃-NE₃, NaHCO₃, NaOH, H₂O, 45%.

Figure S4: ^19F NMR spectrum of purified sulphated metabolite 7.
General

All non-aqueous reactions were performed using flame- or oven dried glassware under an atmosphere of dry nitrogen. All reagents and solvents were purchased from Sigma-Aldrich or Fischer Scientific and were used without further purification. HPLC grade solvents were used for HPLC purification and mass spectrometry grade for UHPLC-ESI-MS analysis. Solutions were concentrated in vacuo on a Heidolph or a IKA rotary evaporator. Thin Layer Chromatography (TLC) was performed on silica gel 60 F254 plates. Visualization of the developed chromatogram was performed using fluorescence quenching or staining with CAM (Cerium Ammonium Molybdate), Ninhydrin, Ehrlich reagent (4-(Dimethylamino)benzaldehyde) or Vanillin. Chromatographic purification of products was accomplished using flash column chromatography on Merck silica gel 60 (40–63 μm) or preparative reverse phase HPLC on an Agilent HPLC-1100 series system equipped with a Waters Atlantis T3 preparative column (10x100 mm, 5 μm) at a 2.5 mL/min flow rate. All synthesized compounds were ≥95% pure as determined by NMR. NMR spectra were recorded on an Agilent 400 MHz spectrometer (1H NMR: 399.97 MHz, 13C NMR: 100.58 MHz) or Varian 300 MHz spectrometer (13C NMR: 75.43 MHz). Chemical shifts are reported in parts per million (ppm) on the δ scale from an internal standard. Multiplicities are abbreviated as follows: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet. High-resolution mass spectra were acquired on a SYNAPT G2-S High Definition Mass Spectrometry (HDMS) using an electrospray ionization (ESI) source with a AQUITY UPLC I-class system and equipped with a Waters ACQUITY UPLC BEH C18 column (2.1 × 75 mm, 1.7 μm particle size) or Waters ACQUITY UPLC HSS T3 column (1.8 × 100 mm, 2.1 μm particle size).

UHPLC-MS/MS analysis

The analysis was performed on an Acquity UPLC system hyphenated with a Synapt G2 Q-TOF, both from Waters Corporation (Milford, MA, USA). The system was controlled using the MassLynx software v 4.1, also from Waters. The separation was performed on a Kinetex biphenyl column (1.7 μm, 100 x 2.1 mm) from Phenomenex (Torrance, CA, USA) and an Acquity BEH C18 column (1.7 μm, 100 x 2.1 mm) from
Waters Corporation. The mobile phase consisted of A) 0.1 % formic acid in MilliQ water and B) methanol and the flow rate was 0.5 mL/min. For the biphenyl column the column temperature was 40 °C and the gradient was 0-1 min, 5% B; 1-11 min, 5-95% B; 11-12 min, 95% B; 12 min, 95-5% B, 12-14 min, 5% B. For the C18 column, the temperature was set to 65 °C and the gradient was 0-1 min, 5% B; 1-6 min, 5-95% B; 6-8 min, 95% B; 8 min, 95-5% B, 8-10 min, 5% B.

The samples were introduced to the q-TOF using negative electrospray ionization. The capillary voltage was set to -2.50 kV and the cone voltage was 30 V. The source temperature was 120 °C, the cone gas flow 50 L/min and the desolvation gas flow 800 L/min. The instrument was operated in MSE mode, the scan range was m/z 50-1200, and the scan time was 0.2 s. In low energy mode, the collision energy was 4 V and in high energy mode the collision energy was ramped between 20-40 V. A solution of sodium formate (0.5 mM in 2-propanol:water, 90:10, v/v) was used to calibrate the instrument and a solution of leucine-encephalin (2 ng/µl in acetonitrile:0.1% formic acid in water, 50:50, v/v) was used for the lock mass correction.

To control the mass accuracy, a known standard mix consisting of leucine-encephalin and meloxicam was analyzed before and after every sample set.

### Chemical Synthesis

**Synthesis of 4-amino-5-hydroxy-2-(trifluoromethyl)benzonitrile (4)**

![Chemical Structure](Image 197x473 to 389x510)

4-Amino-5-methoxy-2-(trifluoromethyl)benzonitrile 13 (35 mg, 160 µmol) was dissolved in DCM (4 mL) and BBr$_3$ (243 µL, 1M solution in heptane, 6 eq) was added dropwise to the reaction mixture at -78 °C. The mixture was stirred at -78 °C and slowly warmed to 25 °C within 4 h. The reaction mixture was then stirred for another 16 h at 25 °C. Upon full consumption of all starting material the reaction mixture was quenched by slow addition of MeOH (2 mL). Silica gel was added to the reaction mixture, the solvent evaporated in vacuo and purified by silica gel chromatography using a gradient of MeOH in DCM (1-5%) to afford 4-amino-5-hydroxy-2-(trifluoromethyl)benzonitrile 4 (11 mg, 54 µmol, 34%).

$^1$H NMR (400 MHz, CD$_3$OD) δ (ppm) = 6.99 (s, 2H); $^{19}$F NMR (376 MHz, CD$_2$OD) δ (ppm) = 62.29 (s, CF$_3$); $^{13}$C NMR (100 MHz, CD$_2$OD) δ (ppm) = 145.7, 142.0, 125.2 (q, $^2$J$_{CF} = 31.9$ Hz), 120.6 (q, $^1$J$_{CF} = 272$ Hz, CF$_3$), 117.7, 116.9, 110.6 (q, $^3$J$_{CF} = 4.8$ Hz), 94.1 (q, $^4$J$_{CF} = 2.4$ Hz); HRMS (ESI-) calculated for C$_{9}$H$_{13}$F$_{3}$N$_{2}$O$^-$ (M-H)$^-$: 201.0281, found: 201.0278.

**Synthesis of 2-amino-5-cyano-4-(trifluoromethyl)phenyl hydrogen sulfate (5)**

![Chemical Structure](Image 202x238 to 404x274)

NaHCO$_3$ (6.7 mg, 79 µmol, 4 eq) and a sulfur trioxide-trimethyl complex (6.9 mg, 50 µmol, 2.5 eq) were added to a solution of 4-amino-5-hydroxy-2-(trifluoromethyl)benzonitrile 4 (4.0 mg, 20 µmol) and NaOH (2.4 mg, 59 µmol, 3 eq) in water (500 µL). The mixture was heated at 40 °C and stirred for 3 days. Upon full consumption of all starting material the solvent was removed and co-evaporated with MeOH. The crude reaction mixture was subjected to HPLC purification [r.t. = 15 min, 0-5 min (0% B), 5-20 min (0-100% B) at a flow of 2.5 mL/min; buffer A = ammonium acetate 5 mM (water) and buffer B = ammonium acetate 5 mM (MeOH)] to afford 2-amino-5-cyano-4-(trifluoromethyl)phenyl hydrogen sulfate 5 (4.0 mg, 14 µmol, 72%).

$^1$H NMR (400 MHz, CD$_2$OD) δ (ppm) = 7.69 (s, 1H), 7.12 (s, 1H); $^{19}$F NMR (376 MHz, CD$_2$OD) δ (ppm) = 63.29 (s, CF$_3$); $^{13}$C NMR (100 MHz, CD$_2$OD) δ (ppm) = 145.8, 139.8, 129.7 (q, $^2$J$_{CF} = 31.8$ Hz), 127.2, 122.8 (q, $^1$J$_{CF} = 272$ Hz, CF$_3$), 116.3, 112.3 (q, $^3$J$_{CF} = 4.8$ Hz), 93.4 (q, $^4$J$_{CF} = 2.1$ Hz); HRMS (ESI-) calculated for C$_{9}$H$_{8}$F$_{3}$N$_{2}$O$^-$S$^-$ (M-H)$^-$: 280.9849, found: 280.9844.
**Synthesis of 2-amino-5-nitro-4-(trifluoromethyl)phenol (6)**

2-Methoxy-4-nitro-5-(trifluoromethyl)aniline 16 (40 mg, 169 µmol) was dissolved in DCM (3 mL) and BBr₃ (254 µL, 1M solution in heptane, 6eq) was added dropwise into the reaction mixture at -78 °C. The mixture was stirred at -78 °C and slowly warmed to 25 °C within 4 h. The reaction mixture was then stirred for another 16 h at 25 °C. Upon full consumption of all starting material the reaction mixture was quenched by slow addition of MeOH (2 mL). Silica gel was added in the reaction mixture. The reaction mixture was evaporated. The crude material was purified by silica gel column by using gradient mixture of 1:5% of MeOH in DCM to afford 2-amino-5-nitro-4-(trifluoromethyl)phenol 6 (15 mg, 88 µmol, 40%).

**Synthesis of 2-amino-5-nitro-4-(trifluoromethyl)phenyl hydrogen sulfate (7)**

Sodium bicarbonate (9.8 mg, 120 µmol, 4 eq) and a sulfur trioxide-trimethyl complex (10 mg, 73 µmol, 2.5 eq) were added to a solution of 2-amino-5-nitro-4-(trifluoromethyl)phenol 6 (6.5 mg, 29 µmol) and NaOH (3.5 mg, 88 µmol, 3 eq) in water (500 µL). Upon full consumption of all starting material the solvent was removed and co-evaporated with MeOH. The crude reaction mixture was subjected to HPLC purification [r.t.= 16 min, 0-5 min (0% buffer B), 5-20 min (0-100% buffer B); buffer A = ammonium acetate 5 mM (water) and buffer B = ammonium acetate 5 mM (MeOH)] to afford 2-amino-5-nitro-4-(trifluoromethyl)phenyl hydrogen sulfate 7 (4 mg, 10 µmol, 45%).

**Synthesis of 4-amino-5-iodo-2-(trifluoromethyl)benzonitrile (12)**

4-Amino-2-(trifluoromethyl)benzonitrile 11 (400 mg, 2.15 mmol) was dissolved in a 1:1 mixture of THF (10 mL) and methanol (10 mL). N-iodosuccinimide (531 mg, 2.36 mmol, 1.1 eq) was then added, followed by addition of about 5 mol % of p-toluenesulfonic acid monohydrate (18.5 mg, 108 µmol). The solution was stirred for 16 h, then concentrated. The crude was dissolved in ethyl acetate (30 mL) and the solution was washed with water twice, then washed with brine and evaporated to afford solid. The crude product was purified by silica gel column chromatography by using gradient mixture of 10-30% EtOAc in hexane to afford 4-amino-5-iodo-2-(trifluoromethyl)benzonitrile 12 (625 mg, 2.00 mmol, 93%).

**References**

1H NMR (400 MHz, CD₂OD) δ (ppm) = 8.11 (s, 1H), 7.08 (s, 1H); 19F NMR (376 MHz, CD₂OD) δ (ppm) = 63.38 (s, CF₃); 13C NMR (100 MHz, CD₂OD) δ (ppm) = 152.7, 145.3, 132.9 (q, J₇CF = 33 Hz), 122.8 (q, J₆CF = 272 Hz, CF₃), 115.1, 110.5 (q, J₅CF = 5.0 Hz), 95.9, 83.1 (q, J₄CF = 1.3 Hz); HRMS (ESI+) calculated for C₇H₅F₃I⁺, [M+H]⁺ = 312.9443, found: 312.9443.
Synthesis of 4-amino-5-methoxy-2-(trifluoromethyl)benzonitrile (13)

4-amino-5-iodo-2-(trifluoromethyl)benzonitrile 12 (150 mg, 481 µmol), Cul (9.2 mg, 48 µmol, 0.1 eq), Cs₂CO₃ (313 mg, 961 µmol, 2 eq) and 1,10-phenanthroline-mono hydrate (17 mg, 96 µmol, 0.2 eq) were mixed in 3 mL of methanol and flushed with nitrogen for 5 minutes. The reaction mixture was heated at 90 °C for 20 h. The solvent was removed and the crude product was purified by silica gel column chromatography by using gradient mixture of 10-30% EtOAc in hexane to afford 4-amino-5-methoxy-2-(trifluoromethyl)benzonitrile 13 (52 mg, 240 µmol, 50%). The analytical data of 13 perfectly agree with reference data.

Synthesis of 2-iodo-4-nitro-5-(trifluoromethyl)aniline (15)

5-Amino-2-nitrobenzotrifluoride 14 (1.00 g, 4.85 mmol) was dissolved in a 1:1 mixture of THF (10 mL) and methanol (10 mL). N-iodosuccinimide (1.20 g, 5.34 mmol, 1.1 eq) were then added, followed by addition of about 5 mol % of p-toluenesulfonic acid monohydrate (41.8 mg, 243 µmol). The solution was stirred for 16 h, then concentrated. The crude was dissolved in ethyl acetate (30 mL) and the solution was washed with water twice, then washed with brine and evaporated to afford solid. The crude product was purified by silica gel column chromatography by using gradient mixture of 10-30% EtOAc in hexane to afford 2-iodo-4-nitro-5-(trifluoromethyl)aniline 15 (1.47 g, 4.41 mmol, 91%).

^1H NMR (400 MHz, CD₂OD) δ (ppm) = 8.43 (s, 1H), 7.11 (s, 1H); ^19F NMR (376 MHz, CD₂OD) δ (ppm) = 61.95 (s, CF₃); ^13C NMR (100 MHz, CD₂OD) δ (ppm) = 153.7, 137.8, 135.5, 125.8 (q, J₉CF = 33 Hz), 122.3 (q, J₉CF = 73 Hz, CF₃), 110.7 (q, J₉CF = 7.0 Hz), 80.9; HR-MS (ESI+) calculated for C₇H₅F₃IN₂O₂ (M-H)^−: 330.9197, found: 330.9193.

Synthesis of 2-methoxy-4-nitro-5-(trifluoromethyl)aniline (16)

2-Iodo-4-nitro-5-(trifluoromethyl)aniline 15 (60 mg, 180 µmol), Cul (3.4 mg, 18 µmol, 0.1 eq), Cs₂CO₃ (118 mg, 361 µmol, 2 eq) and 1,10-phenanthroline-mono hydrate (6.5 mg, 36 µmol, 0.2 eq) were mixed in 3 mL of methanol and flushed with nitrogen for 5 minutes. The reaction mixture was heated at 90 °C for 20 h. The solvent was removed and the crude product was purified by silica gel column chromatography by using gradient mixture of 10-30% EtOAc in hexane to afford 2-methoxy-4-nitro-5-(trifluoromethyl)aniline 16 (12.3 mg, 52.1 µmol, 29%) and 4-nitro-3-(trifluoromethyl)aniline (16.3 mg, 78 µmol, 43%).

^1H NMR (400 MHz, CD₂OD) δ (ppm) = 7.61 (s, 1H), 7.05 (s, 1H), 3.97 (s, 3H, OMe); ^19F NMR (376 MHz, CDCl₃) δ (ppm) = 59.29 (s, CF₃); ^13C NMR (100 MHz, CDCl₃) δ (ppm) = 146.9, 140.9, 122.4 (q, J₉CF = 273 Hz, CF₃), 119.0 (q, J₉CF = 33.8 Hz), 111.4 (q, J₉CF = 6.3 Hz), 108.4, 107.6, 56.3; HRMS (ESI-) calculated for Cs₈H₆F₃NO₃ (M-H)^−: 235.0336, found: 235.0329.
References


NMR Spectra

4-Amino-5-hydroxy-2-(trifluoromethyl)benzonitrile (4)
$^1$H NMR, $^{13}$C NMR, $^{19}$F NMR

2-Amino-5-cyano-4-(trifluoromethyl)phenyl hydrogen sulfate (5)
$^1$H NMR, $^{13}$C NMR, $^{19}$F NMR

2-Amino-5-nitro-4-(trifluoromethyl)phenol (6)
$^1$H NMR, $^{13}$C NMR, $^{19}$F NMR

2-Amino-5-nitro-4-(trifluoromethyl)phenyl hydrogen sulfate (7)
$^1$H NMR, $^{13}$C NMR, $^{19}$F NMR

4-Amino-5-iodo-2-(trifluoromethyl)benzonitrile (12)
$^1$H NMR, $^{13}$C NMR, $^{19}$F NMR

2-Iodo-4-nitro-5-(trifluoromethyl)aniline (15)
$^1$H NMR, $^{13}$C NMR, $^{19}$F NMR

2-Methoxy-4-nitro-5-(trifluoromethyl)aniline (16)
$^1$H NMR, $^{13}$C NMR, $^{19}$F NMR
BN1901_19F_No 4

\[ f_1 \ (ppm) \]

\[ -62.29 \]

Chemical Structure:

- NC
- HO
- F_3C
- NH_2
BL2829_13C_No 5

![NMR spectrum](image)

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f1 (ppm)

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BL2829_13C_No 5

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BL3276_19F_No 6

\[ f_1 \text{ (ppm)} \]

-60.55
BN1901_1H_No 7

1.18  1.00  3.30  4.84  7.15  8.14