Evidence of enzyme-mediated transesterification of synthetic cannabinoids with ethanol: potential toxicological impact

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Supplementary data

S1: Synthetic and Analytical Chemistry data of synthesised compounds **2**

S2: Full MS and MS/MS data of ethyl esters and ester hydrolysis products of PB-22, NPB-22, 5F-PB-22, and 5F-NPB-22 incubations with HLM **19**

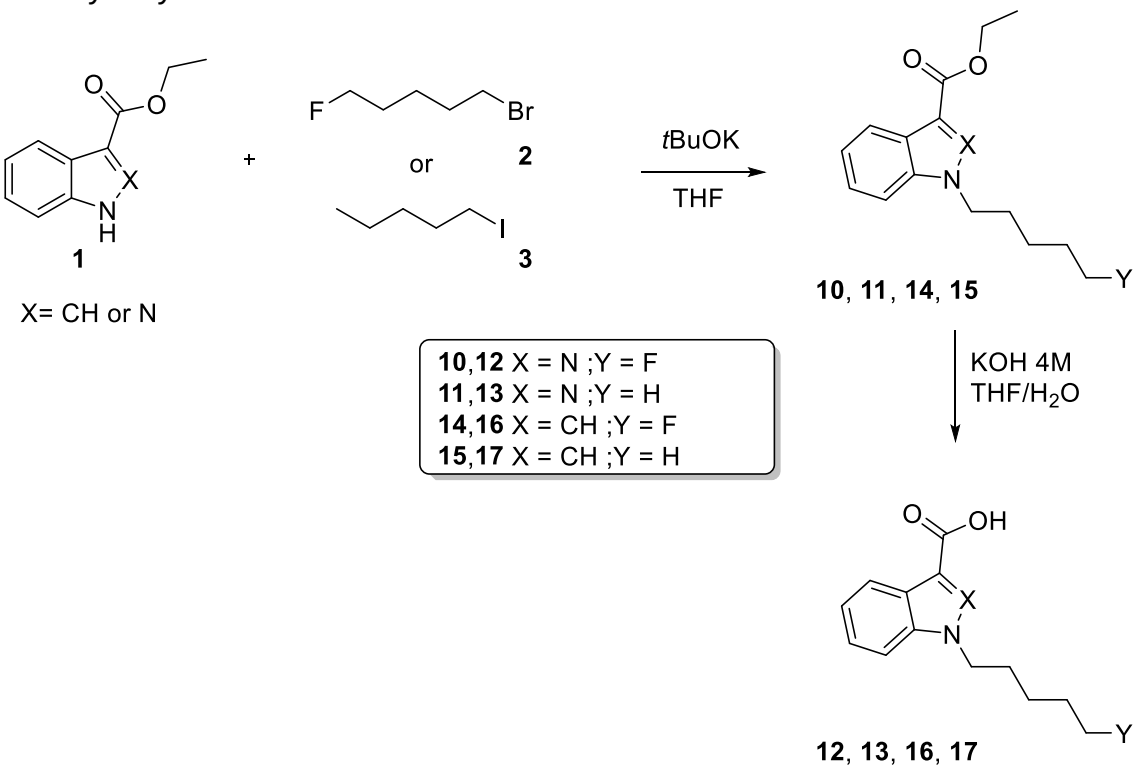
S3: Overlaid extracted ion chromatograms of the major detected metabolites in the presence of ethanol, ethanol and inhibitor and negative control (without HLM) **23**

S4: Proposed metabolic pathway of PB-22, NPB-22, 5F-PB-22, and 5F-NPB-22 in the presence of ethanol **33**

S5: Biological evaluation of PB-22, NPB-22, 5F-PB-22, 5F-NPB-22, their ethyl esters and their ester hydrolysis products **36**

S1: Synthetic and Analytical Chemistry data

The synthesis of the compounds **10**–**17** are reported in the following scheme. Compounds **10**, **11, 14, 15** were synthesized by the nucleophilic substitution of the N1 nitrogen of the commercially available ethyl indazole carboxylate (**1**) and the 1-bromo-5-fluoropentane (**2**) or the 1,5-diiodopentane (**3**). The reaction was carried out in THF using 1.1 eq. of *t*BuOK as a base. Compounds **12**, **13, 16, 17** were synthesized by the hydrolysis of **10**, **11, 14, 15**. The ester hydrolysis were made in THF/KOH 4M.



Scheme S1. Synthesis of the compounds.

*General procedure for the synthesis of compounds **10**, **11**, **14**, **15**.* To a stirred solution of ethyl indazole-3-carboxylate (100 mg, 0.52 mmol) or ethyl indole-3-carboxylate (100 mg, 0.53 mmol) in 3 mL of THF at 0 °C was added 1.1 eq. of tBuOK. After 5 minutes at 0 °C 1 eq. of 1-bromo-5-fluoropentane (**2**) or 1,5-diiodopentane (**3**) was added. The mixture was stirred for 12 h at room temperature and monitored by TLC. The solvent was removed, and the crude of the reaction was purified by chromatography (hexane/EtOAc, 7.5/2.5).

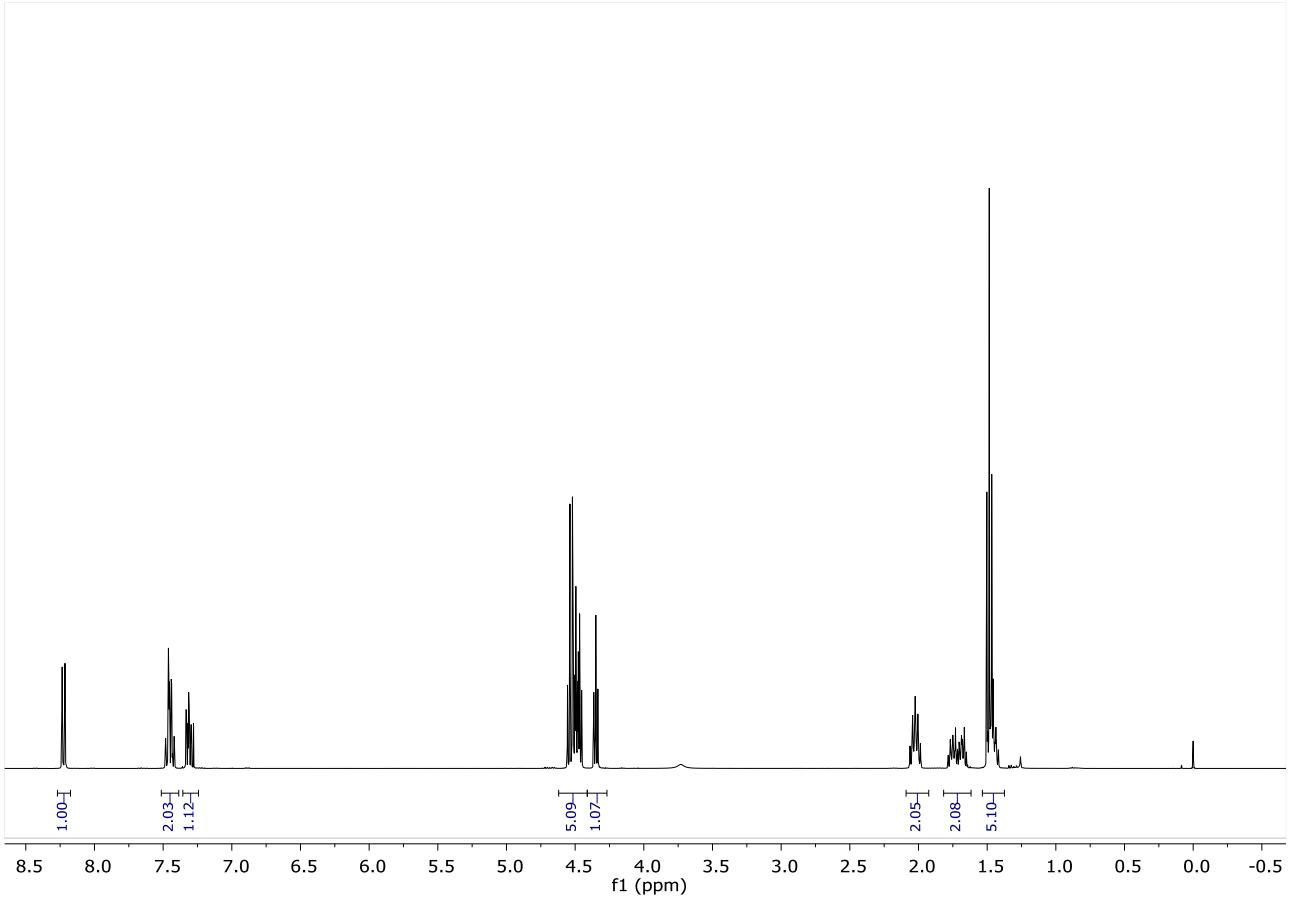
Ethyl esters: to a stirred solution of ethyl indazole-3-carboxylate (100 mg, 0.52 mmol/L) or ethyl indole-3-carboxylate (100 mg, 0.53 mmol/L) in 3 mL of THF at 0 °C was added 1.1 eq. of tBuOK. After 5 minutes at 0 °C 1 eq. of 1-bromo-5-fluoropentane (2) or 1,5-diiodopentane (3) was added. The mixture was stirred for 12 h at room temperature and monitored by TLC. The solvent was removed, and the crude of the reaction was purified by chromatography (hexane/EtOAc, 7.5/2.5).

Hydrolysed esters: 0.52 mmol/L of the precursor compound (**10**, **11**, **14** or **15**) was added to a 4 mol/L solution of KOH in THF (2 mL). The mixture was stirred for 12 to 15 h and monitored by TLC. At the end of the reaction, the solvent was removed. Water and EtOAc were added and the organic phase was collected after the pH of the organic phase was adjusted to 5–6. The organic phase was evaporated, and the crude of the reaction was purified by chromatography (hexane/EtOAc, 7/3).

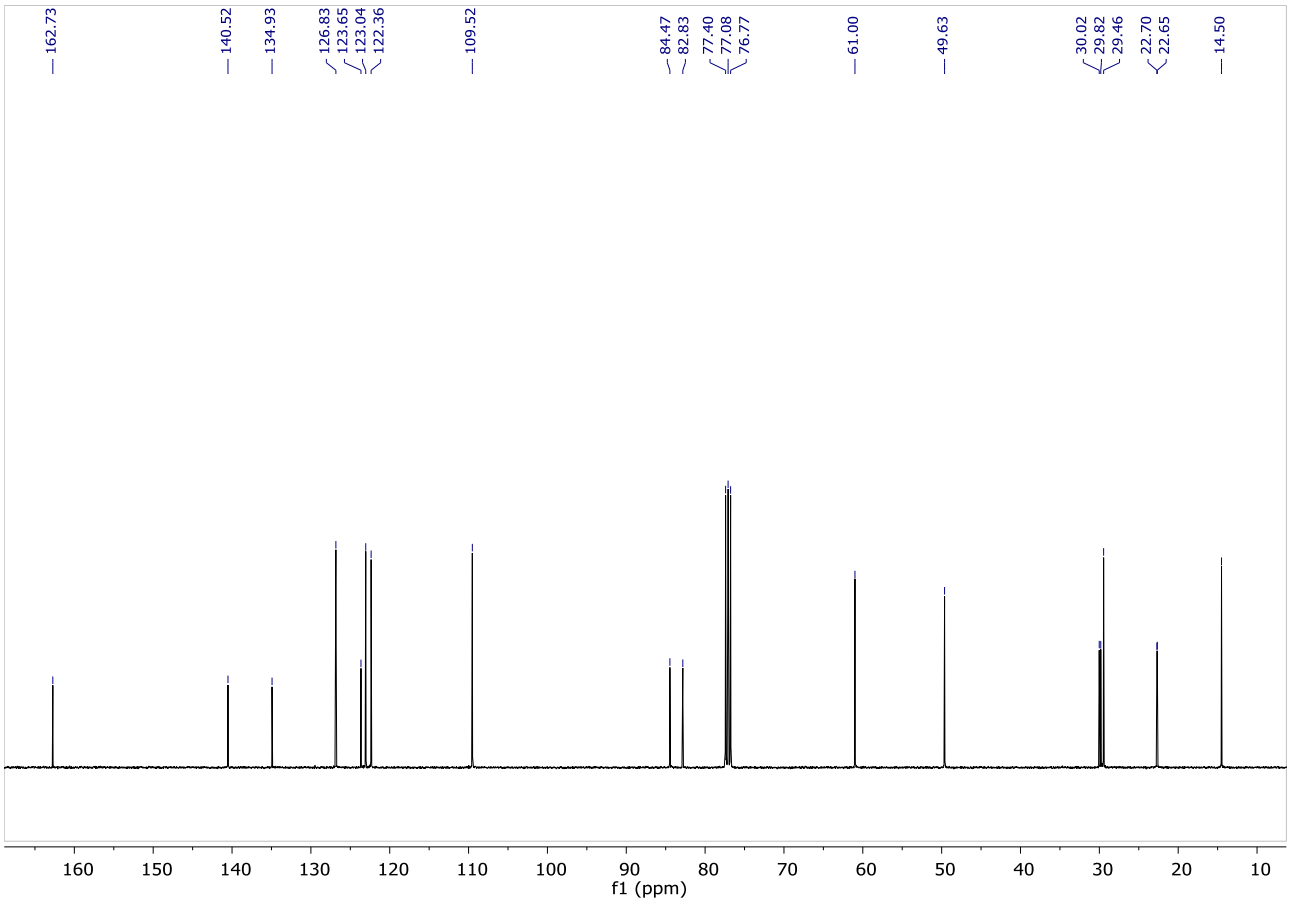
10, pale yellow oil. ^{1}H NMR (400 MHz, Chloroform-*d*) δ = 1.47 (dt, *J*=12.1, 7.7, 5H), 1.62 – 1.82 (m, 2H), 2.02 (p, *J*=7.7, 2H), 4.35 (t, *J*=6.0, 1H), 4.41 – 4.62 (m, 5H), 7.31 (ddd, *J*=8.2, 6.0, 1.5, 1H), 7.39 – 7.51 (m, 2H), 8.23 (dt, *J*=8.2, 1.0, 1H). ^{13}C NMR (101 MHz, $CDCl_3$) δ = 14.50, 22.65, 22.70, 29.46, 29.62, 29.82, 30.02, 49.63, 61.00, 82.83, 84.47, 109.52, 122.36, 123.04, 123.65, 126.83, 134.93, 140.52, 162.73. **11**, pale yellow oil. ^{1}H NMR (400 MHz, Chloroform-*d*) δ = 0.88 (t, *J*=6.9, 3H), 1.18 – 1.42 (m, 4H), 1.48 (t, *J*=6.9, 3H), 1.89 – 2.03 (m, 2H), 4.40 – 4.59 (m, 4H), 7.31 (ddd, *J*=7.9, 6.5, 1.3, 1H), 7.38 – 7.51 (m, 2H), 8.22 (dt, *J*=8.2, 1.3, 1H). ^{13}C NMR (101 MHz, $CDCl_3$) δ = 13.88, 14.51, 22.08, 22.25, 28.91, 29.58, 49.92, 60.96, 109.63, 122.31, 122.96, 123.66, 126.70, 134.73, 140.48, 162.77. **14**, pale yellow oil. 1H NMR (400 MHz, Chloroform-d) δ = 1.44 (dt, J=10.4, 7.3, 5H), 1.55 – 1.80 (m, 3H), 1.92 (dt, J=15.1, 7.3, 2H), 4.16 (t, J=7.3, 2H), 4.29 – 4.41 (m, 3H), 4.41 – 4.52 (m, 1H), 7.21 – 7.33 (m, 2H), 7.33 – 7.41 (m, 1H), 7.82 (s, 1H), 8.11 – 8.26 (m, 1H). 13C NMR (101 MHz, CDCl3) δ = 14.62, 22.81, 22.86, 29.59, 29.72, 29.85, 30.05, 46.86, 53.44, 59.69, 82.83, 84.47, 107.37, 109.88, 121.80, 121.87, 122.68, 126.74, 134.13, 136.47, 165.19. **15**, pale yellow oil. ^{1}H NMR (400 MHz, Chloroform-*d*) δ = 0.81 (t, *J*=6.9, 3H), 1.26 (dqd, *J*=15.2, 7.2, 4.3, 4H), 1.35 (t, *J*=7.2, 3H), 1.81 (q, *J*=7.2, 2H), 4.06 (t, *J*=7.2, 2H), 4.31 (q, *J*=7.2, 2H), 7.09 – 7.25 (m, 2H), 7.25 – 7.33 (m, 1H), 7.75 (s, 1H), 8.11 (ddt, *J*=6.3, 4.1, 2.0, 1H). ^{13}C NMR (101 MHz, $CDCl_3$) δ = 13.91, 14.63, 22.28, 28.99, 29.61, 47.01, 59.65, 107.17, 109.96, 121.71, 121.81, 122.56, 126.73, 134.23, 136.52, 165.25.

*General procedure for the synthesis of compounds **12**, **13**, **16, 17**.* 0.52 mmol of the precursor compound (**10**, **11**, **14** or **15**) was added to a 4 M solution of KOH in THF (2 mL). The mixture was stirred for 12 to 15 h and monitored by TLC. At the end of the reaction, the solvent was removed. Water and EtOAc were added and the organic phase was collected after the pH of the organic phase was adjusted to 5–6. The organic phase was evaporated, and the crude of the reaction was purified by chromatography (hexane/EtOAc, 7/3). NMR data was consistent with the ones previously reported [1-4].

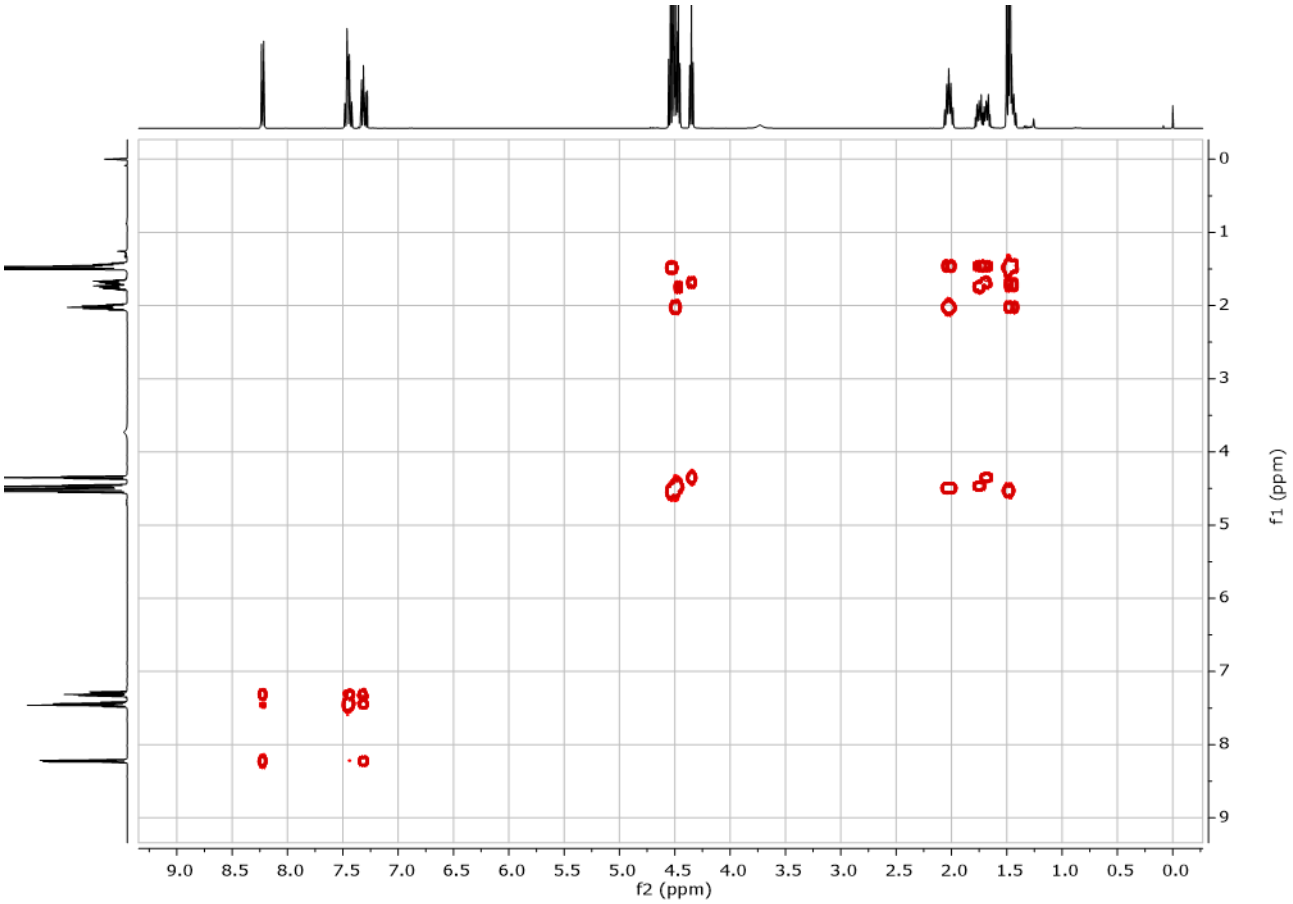
S1.1 ^{1}H NMR of compound 10



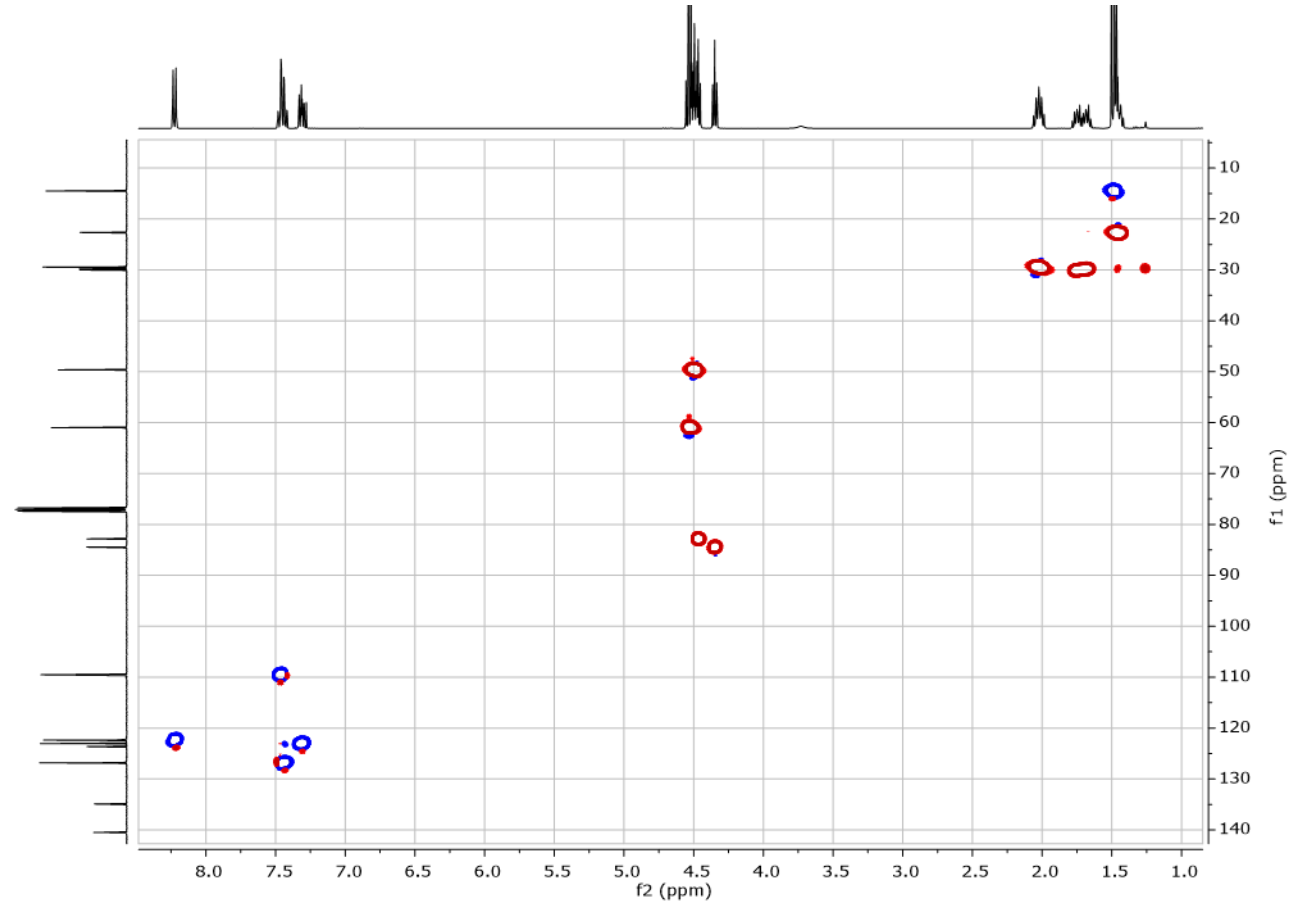
S1.2 ^{13}C NMR of compound 10



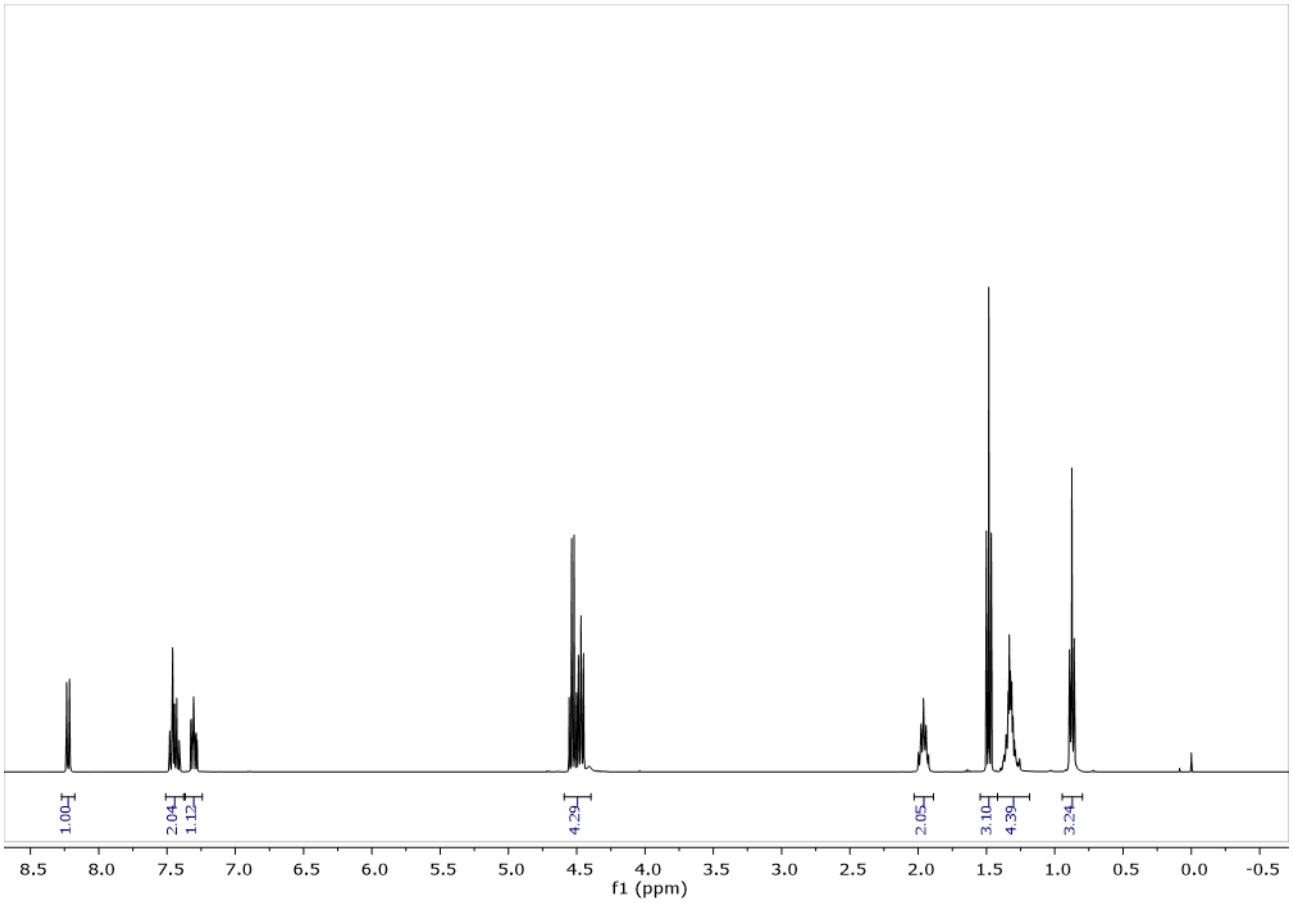
S1.3 COSY of compound 10



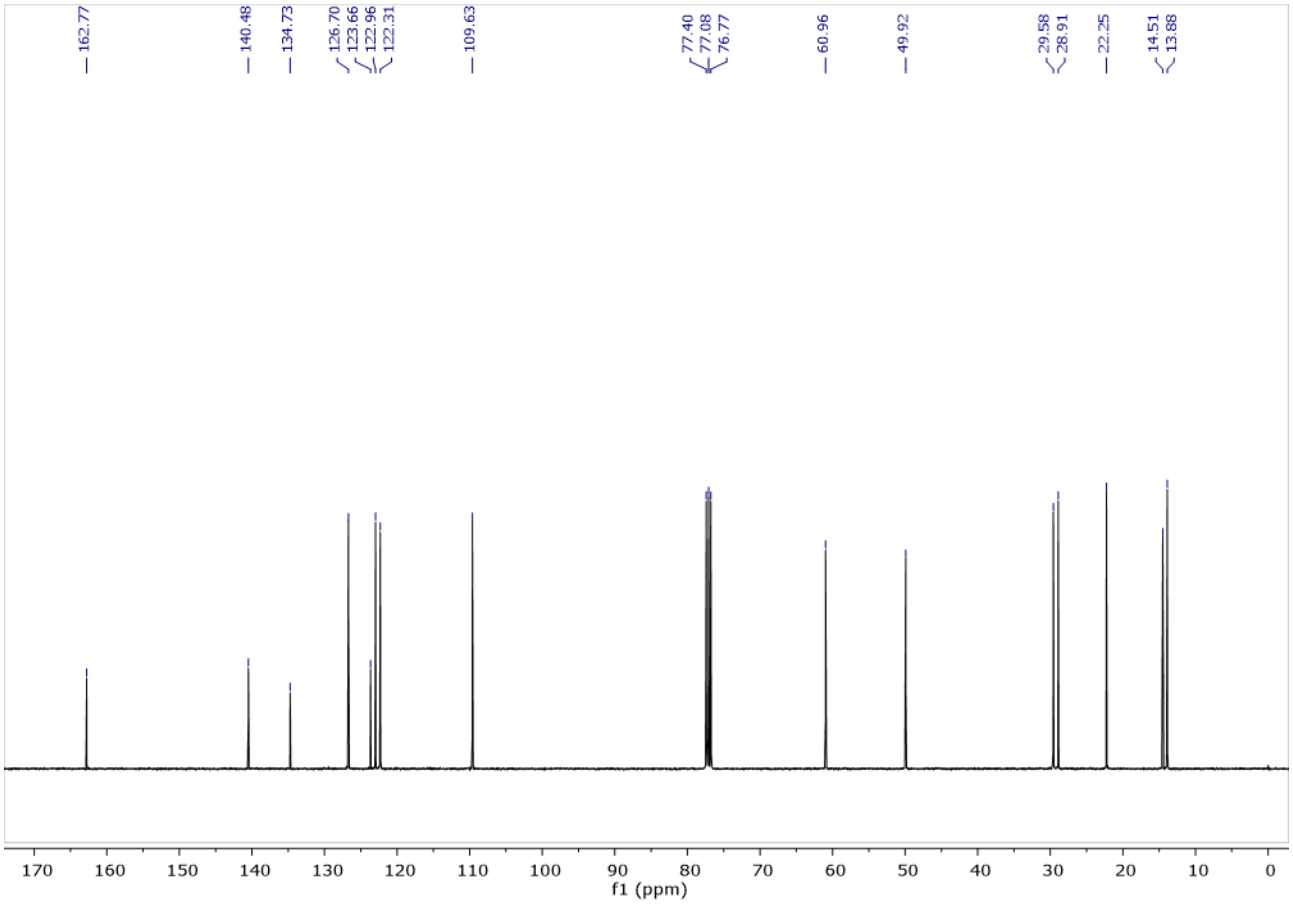
S1.4 HSQC of compound 10



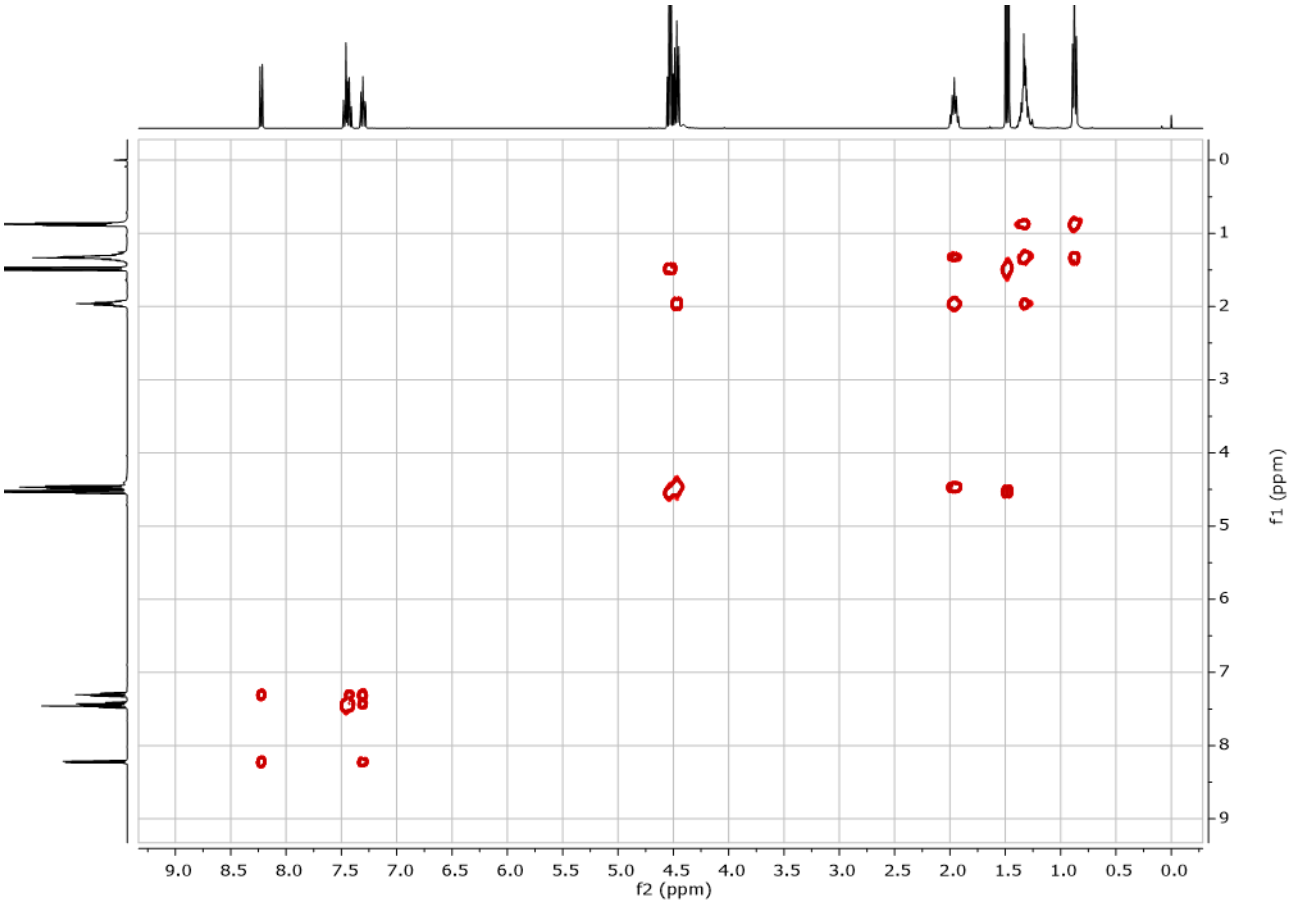
S1.5 ^{1}H NMR of compound 11



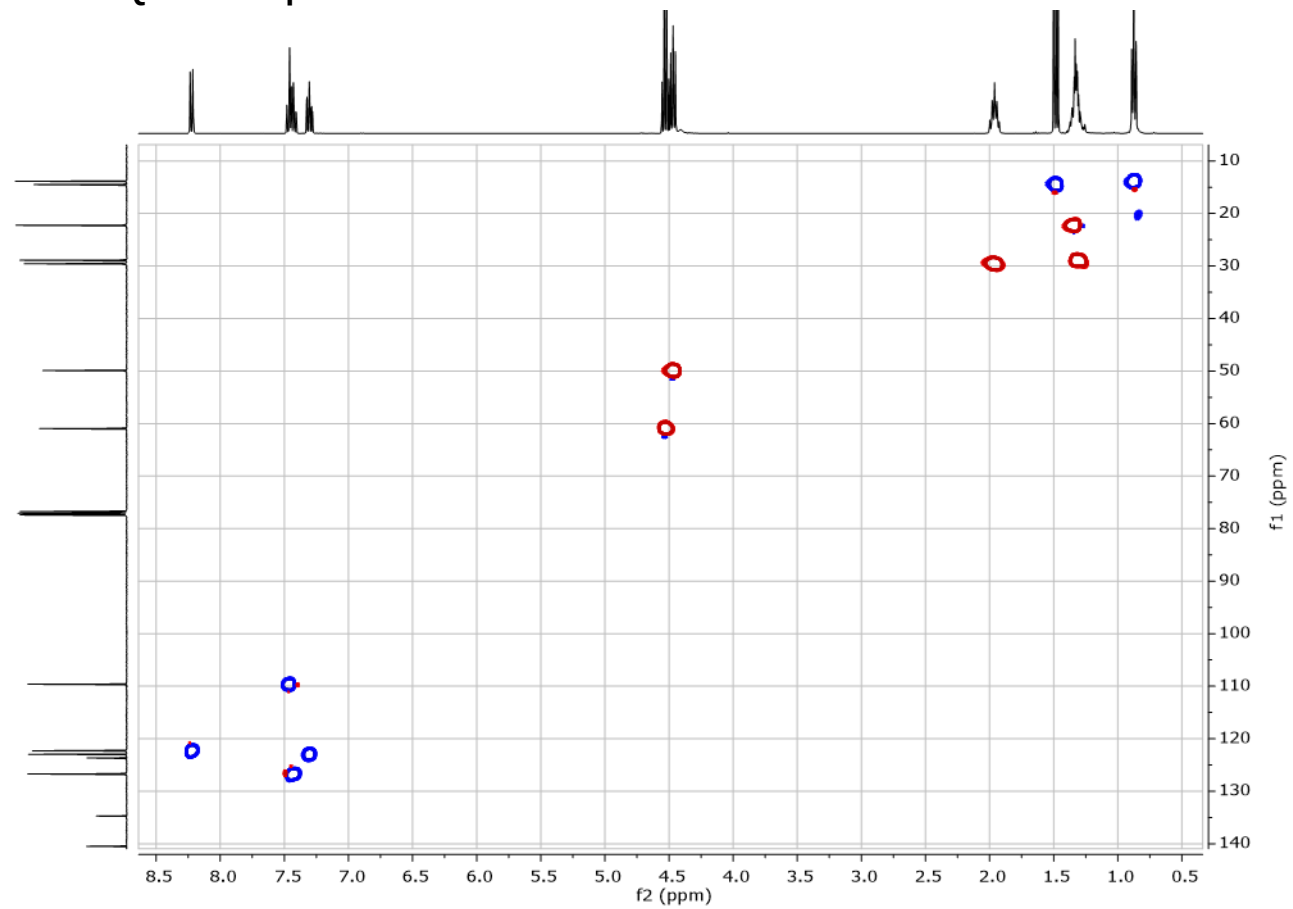
S1.6 ^{13}C NMR of compound 11



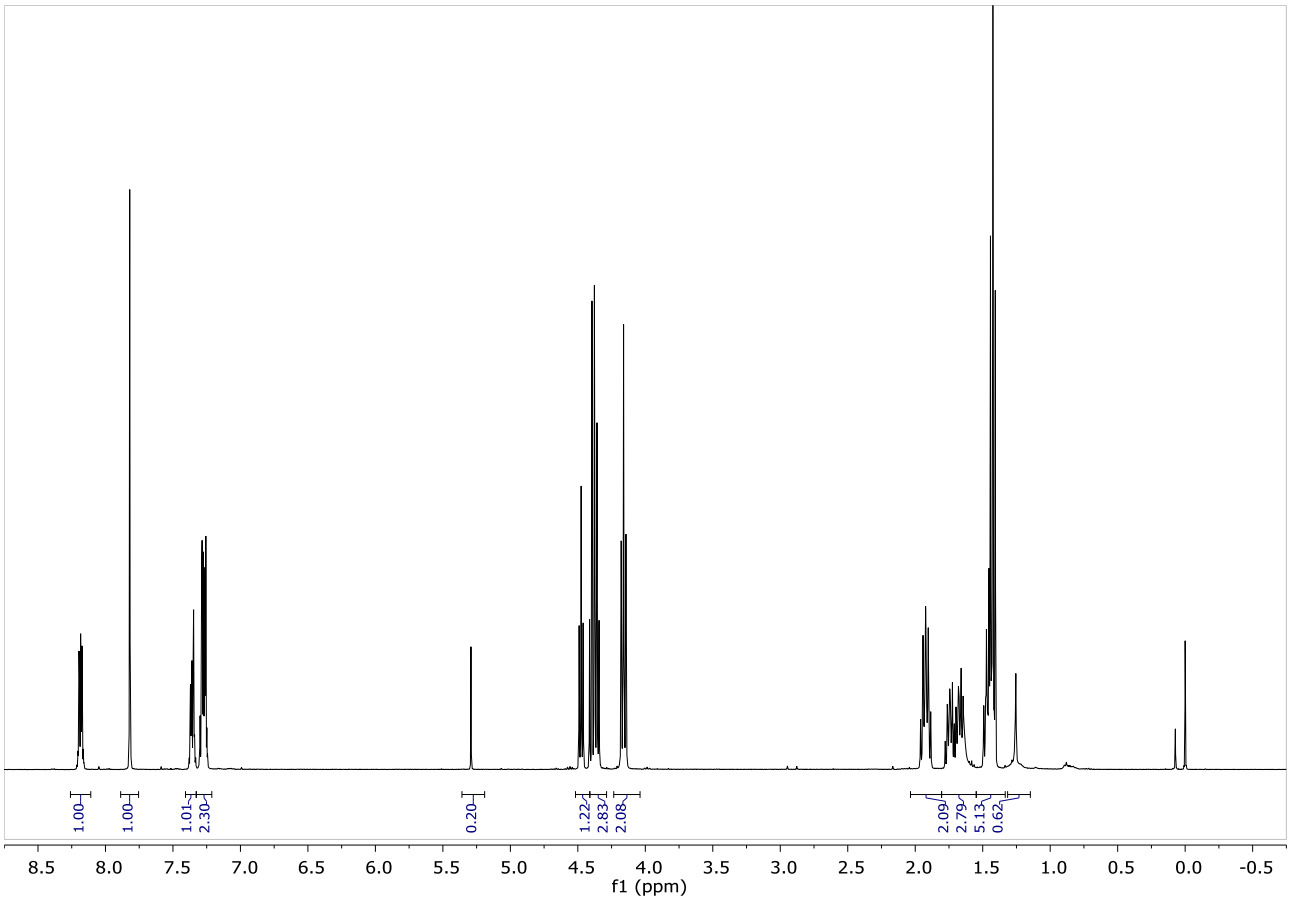
S1.7 COSY of compound 11



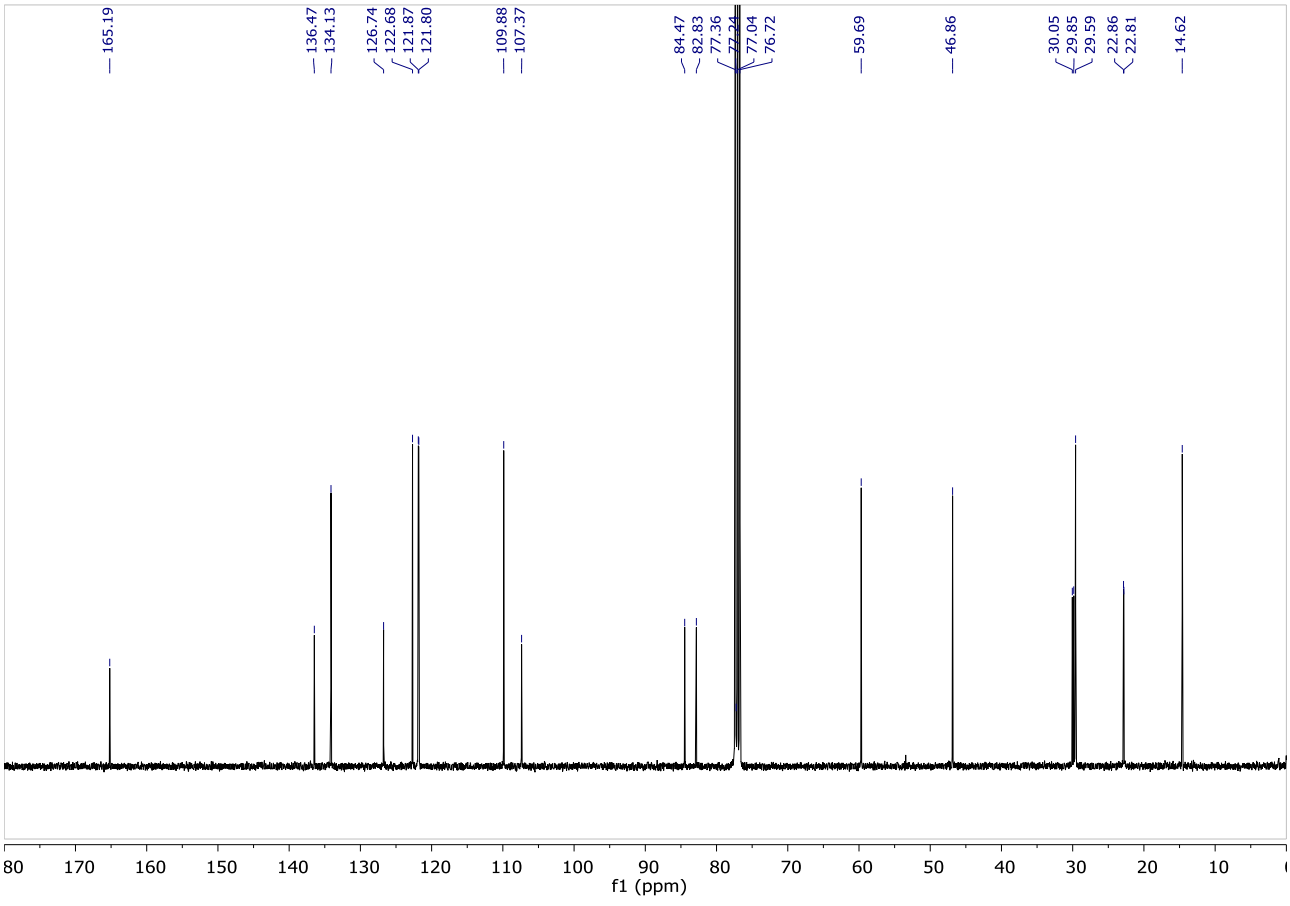
S1.8 HSQC of compound 11



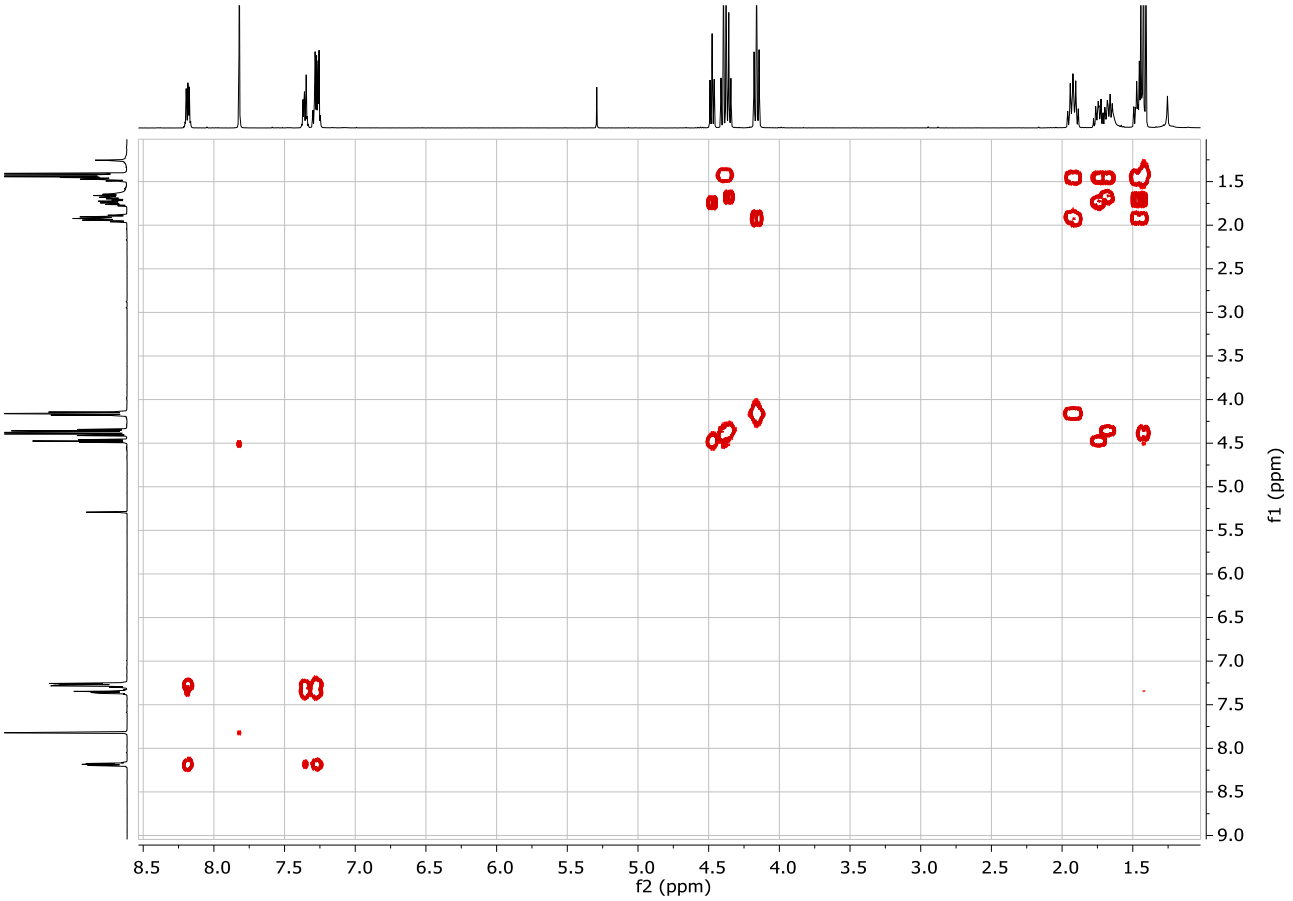
S1.9 ^{1}H NMR of compound 14



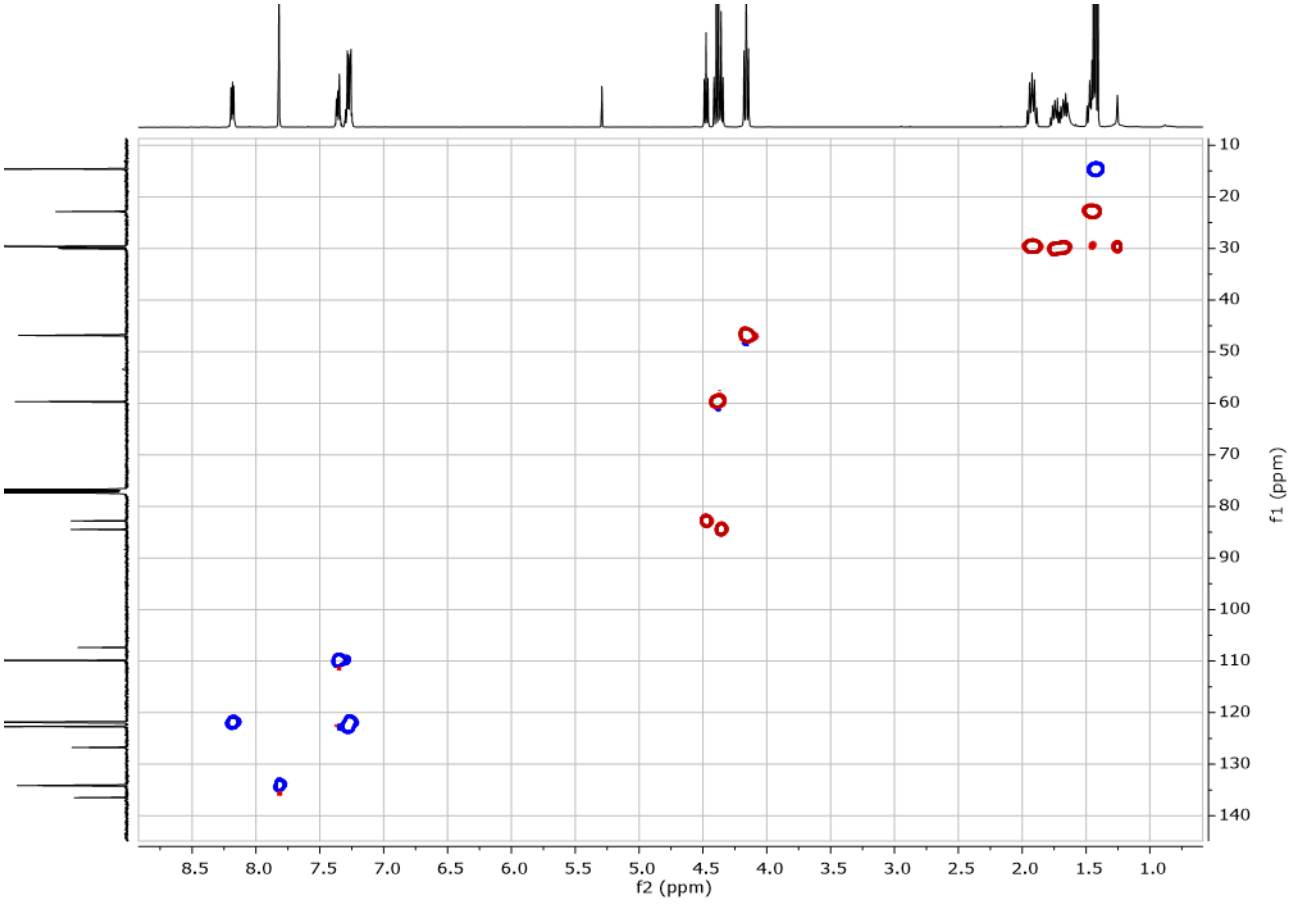
S1.10 ^{13}C NMR of compound 14



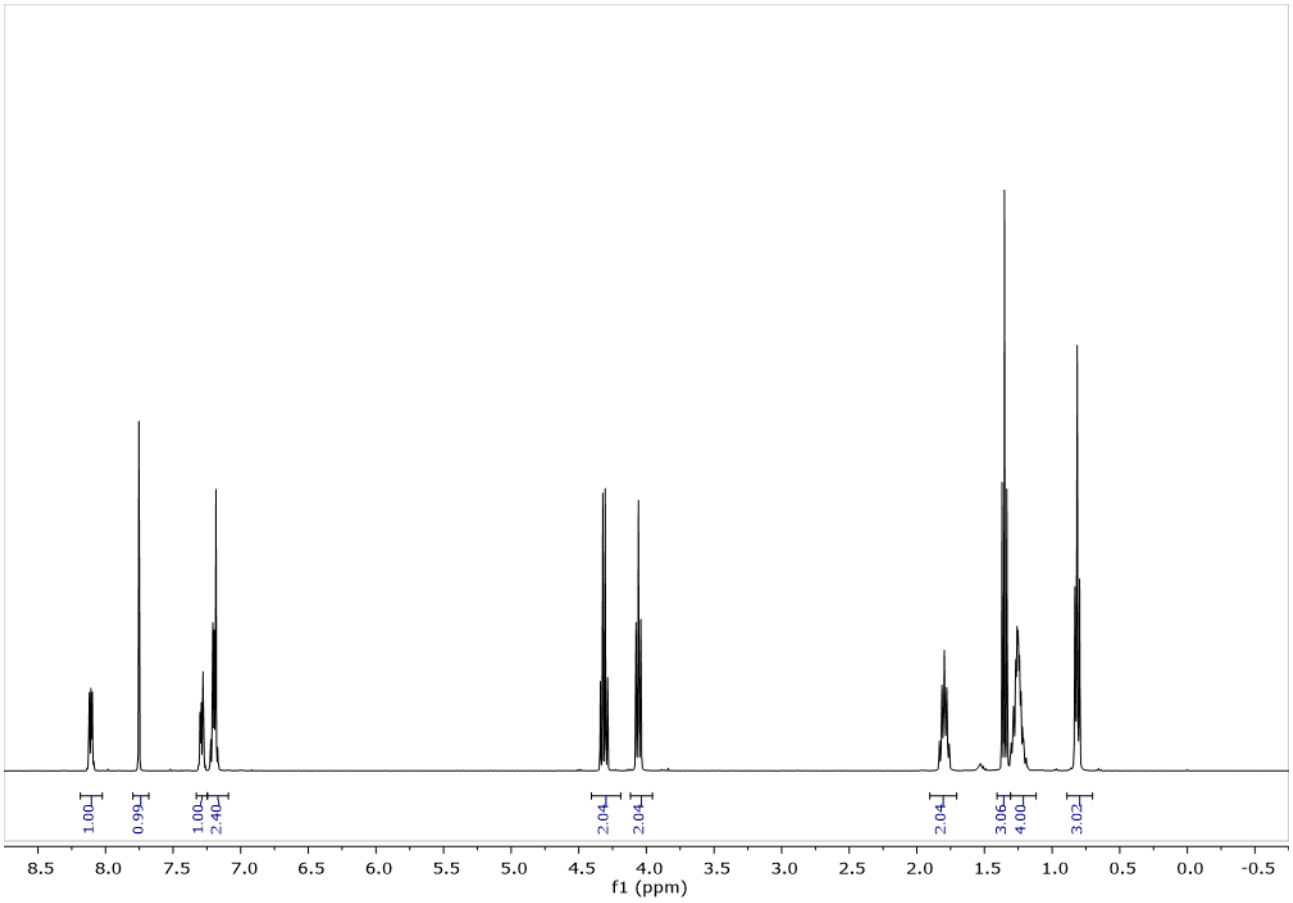
S1.11 COSY of compound 14



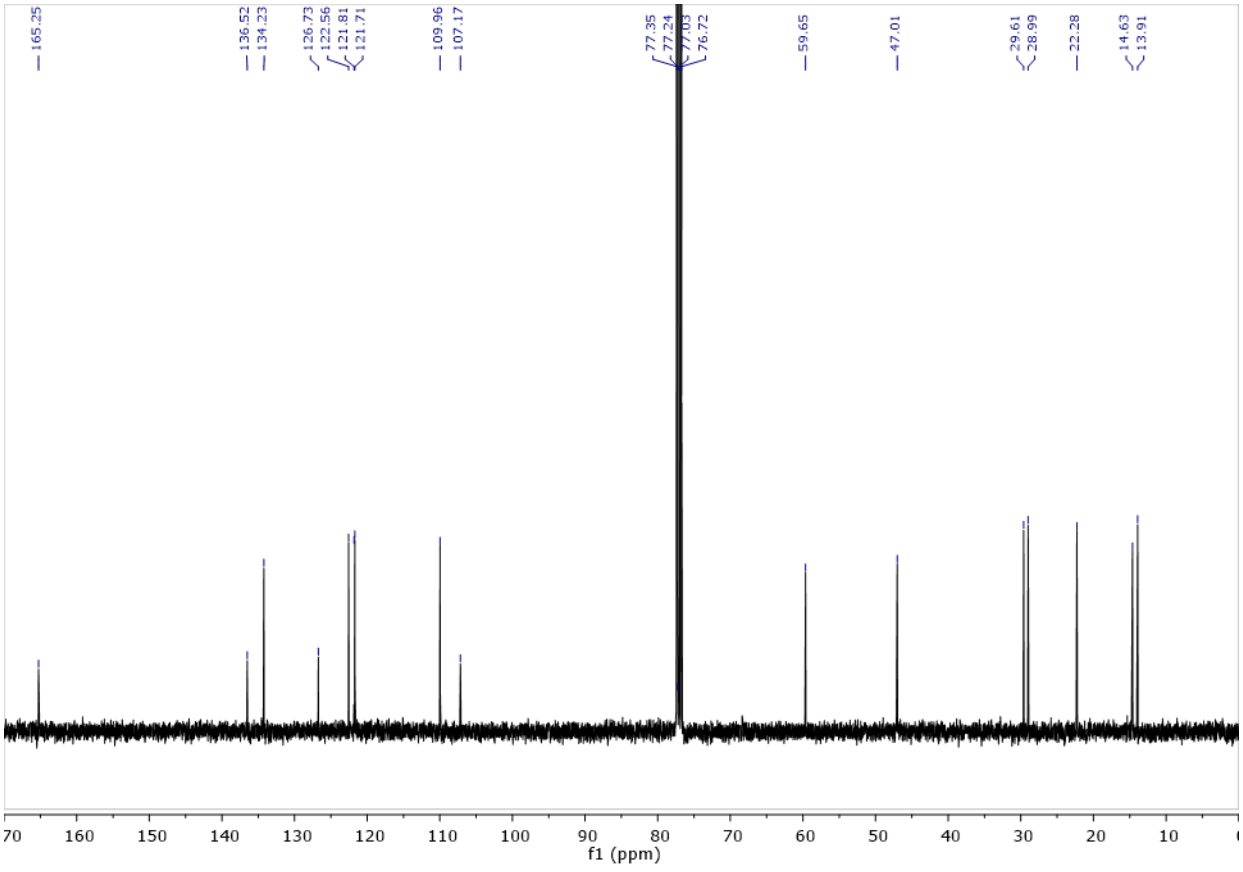
S1.12 HSQC of compound 14



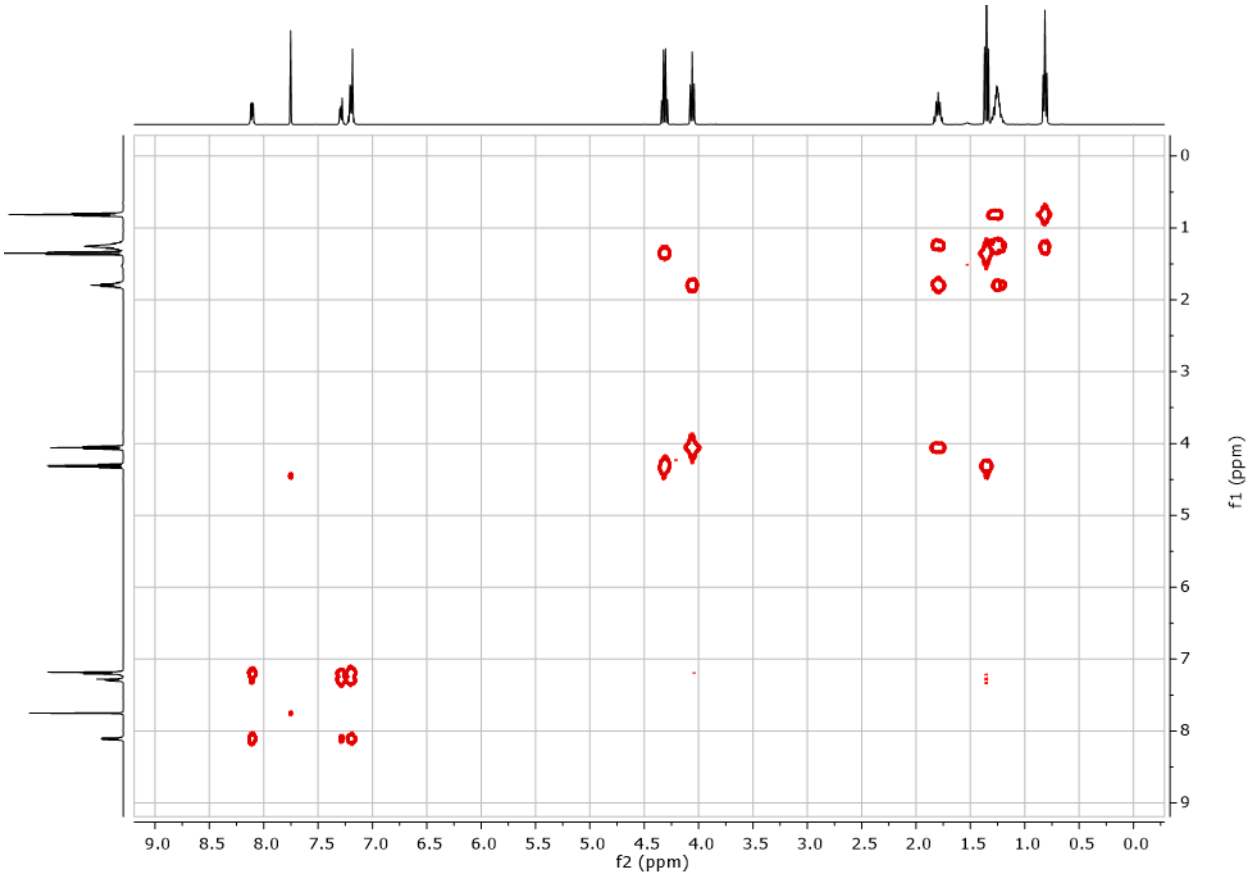
S1.13 ^{1}H NMR of compound 15



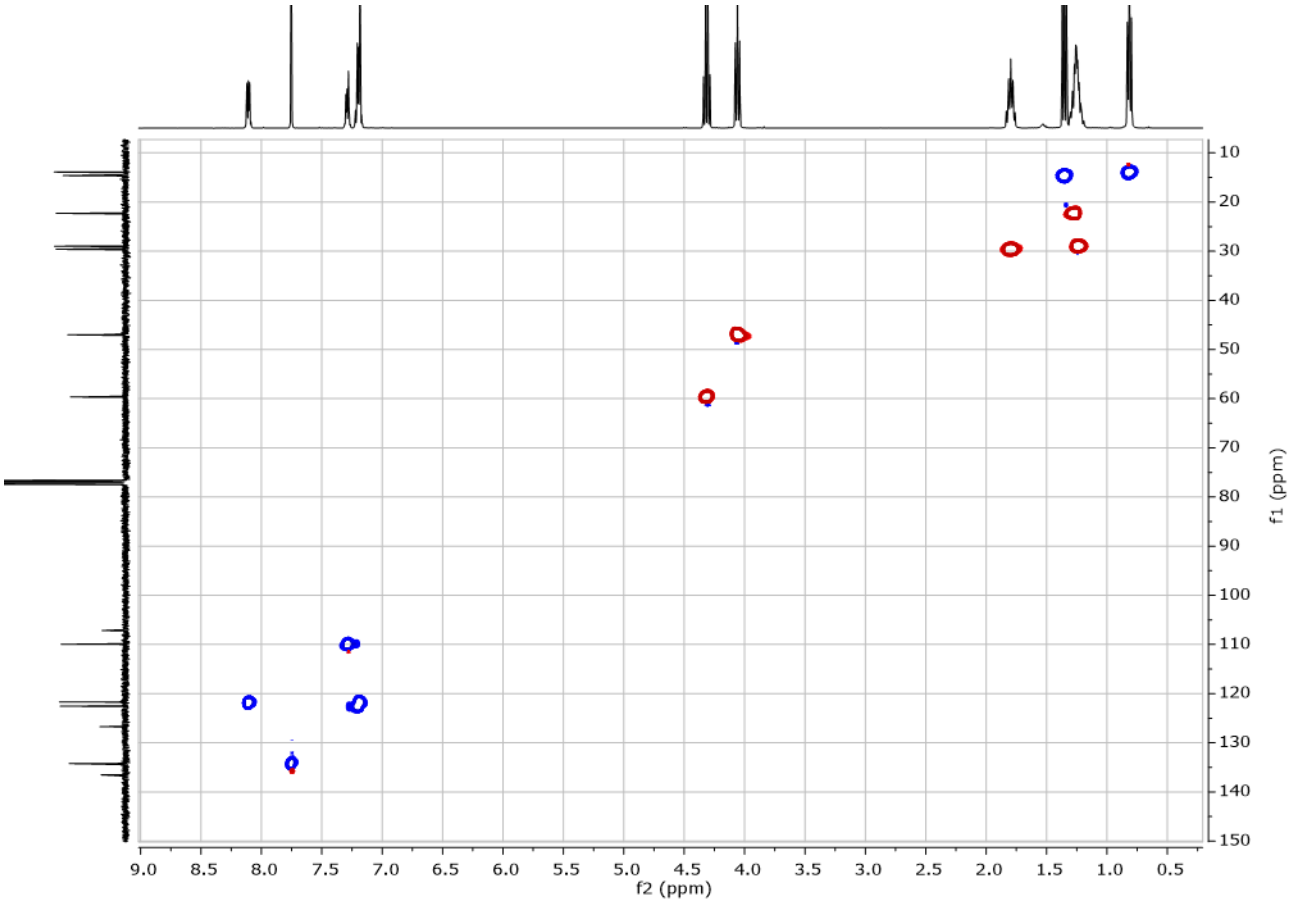
S1.14 ^{13}C NMR of compound 15



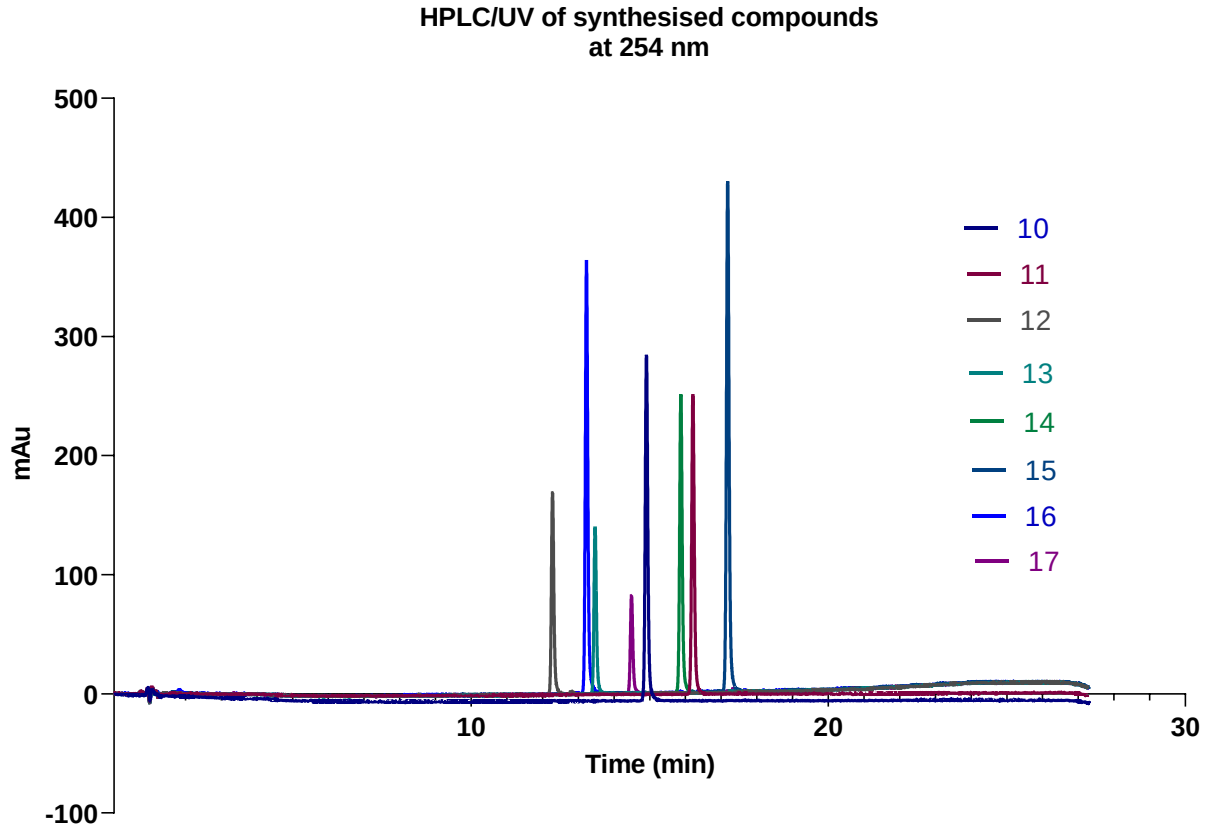
S1.15 COSY of compound 15



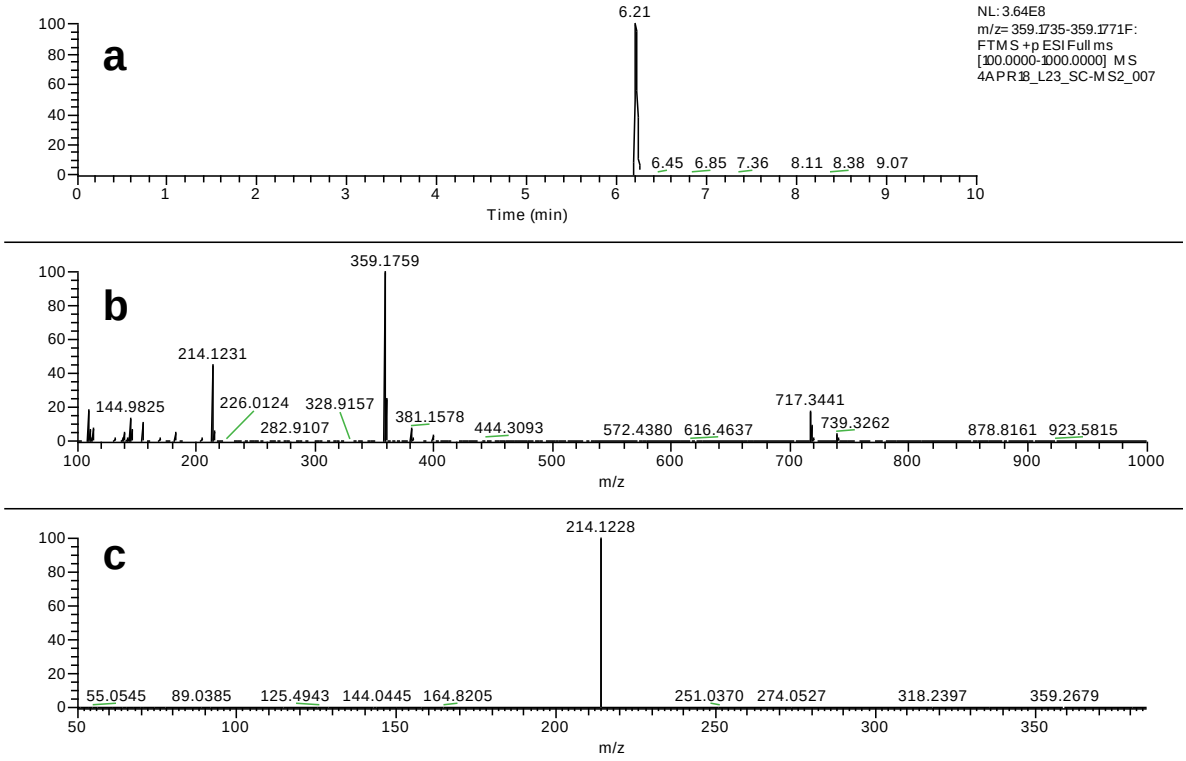
S1.16 HSQC of compound 15



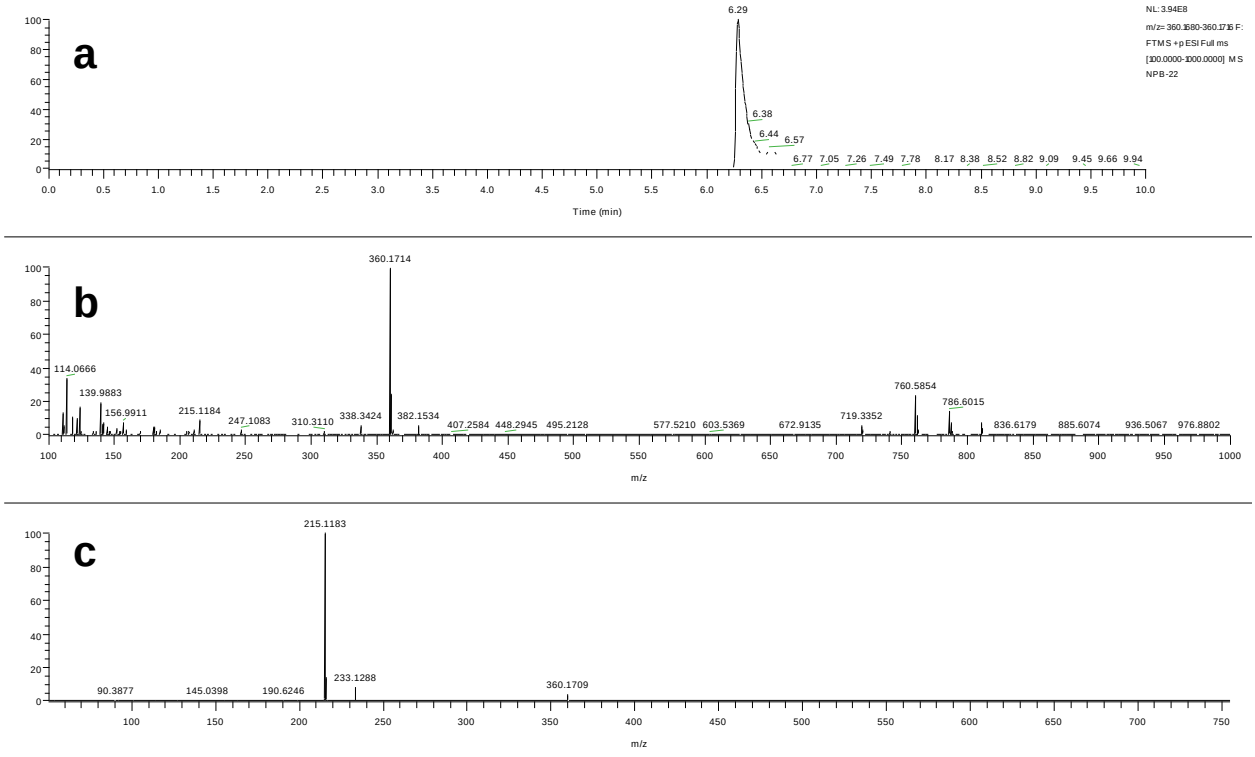
S1.17 HPLC/UV of synthesised compounds (10-17)



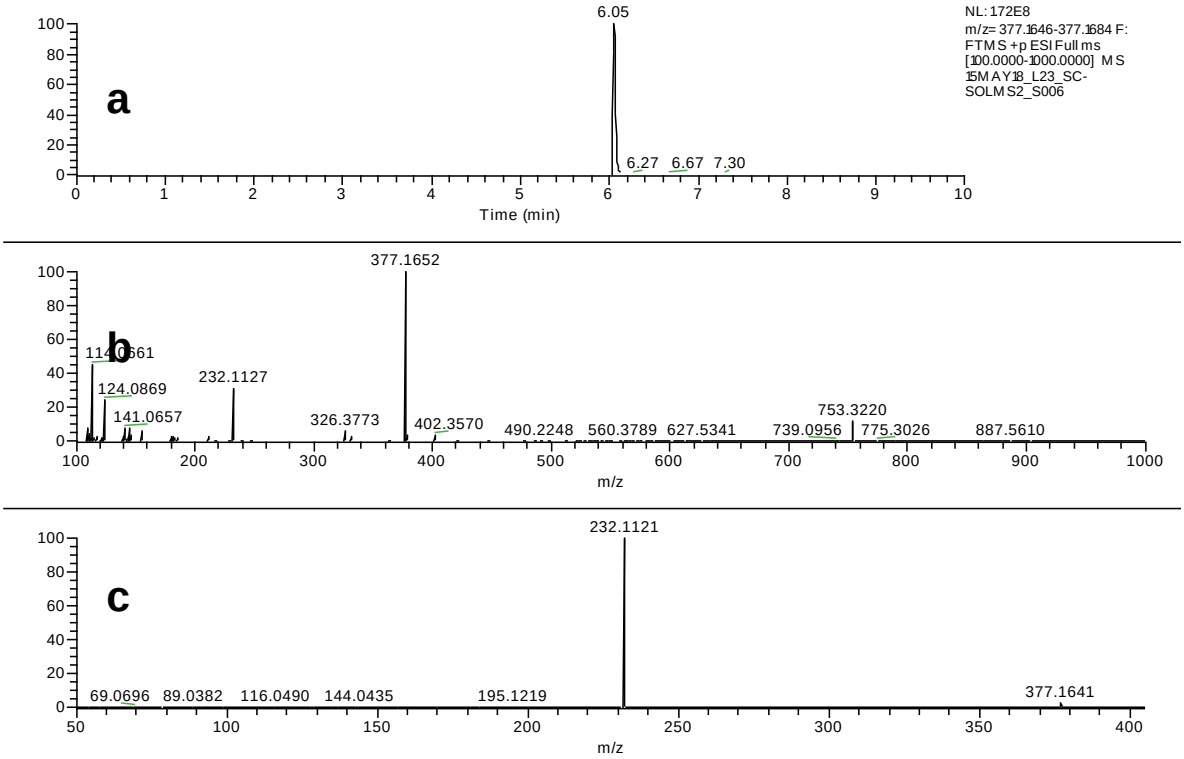
S1.24 LC-HRMS (a), full scan MS spectrum (b) and MS^2 at NCE 10 (c) of PB-22



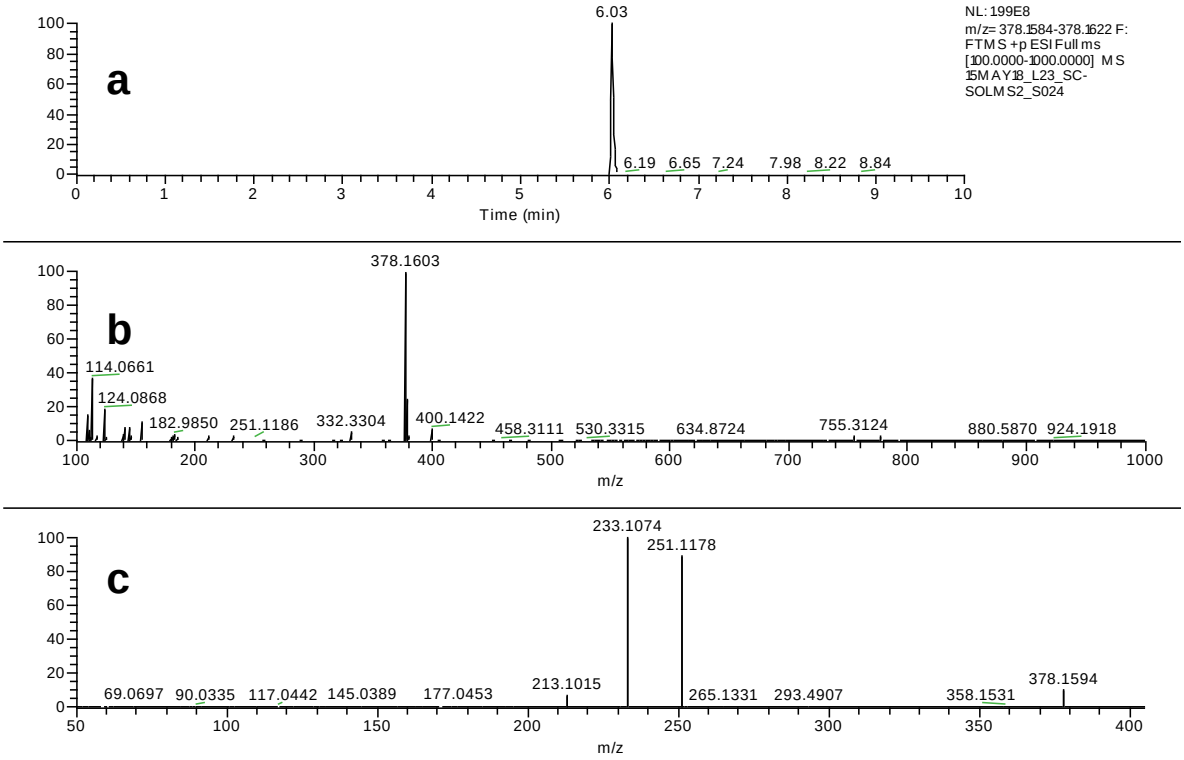
S1.25 LC-HRMS (a), full scan MS spectrum (b) and MS^2 at NCE 20 (c) of NPB-22



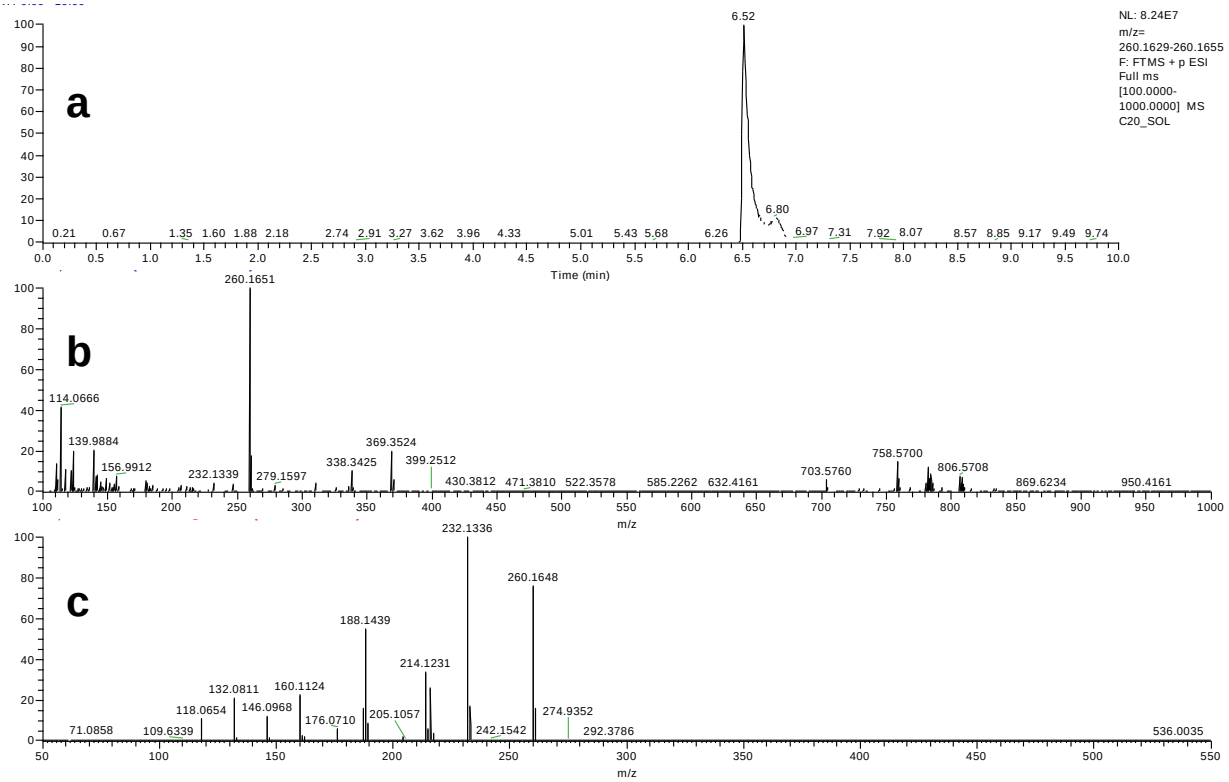
S1.26 LC-HRMS (a), full scan MS spectrum (b) and MS^2 at NCE 13 (c) of 5F-PB-22



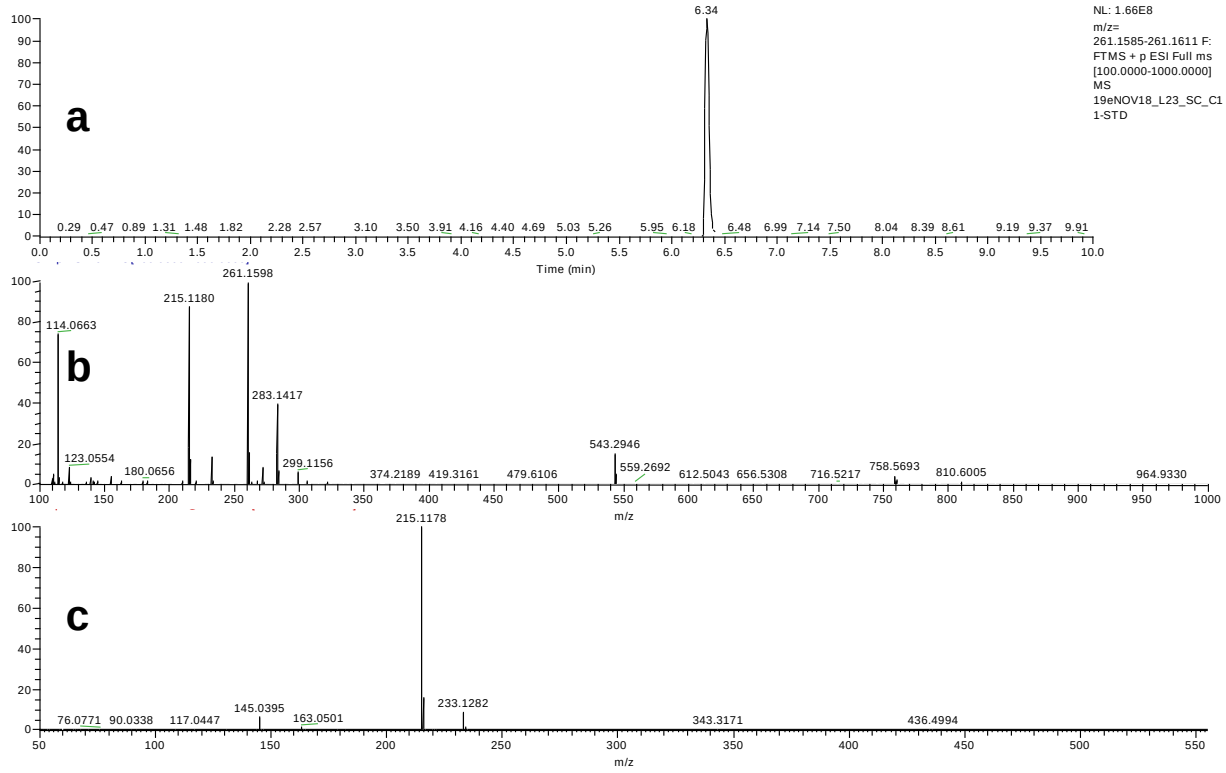
S1.27 LC-HRMS (a), full scan MS spectrum (b) and MS^2 at NCE 20 (c) of 5F-NPB-22



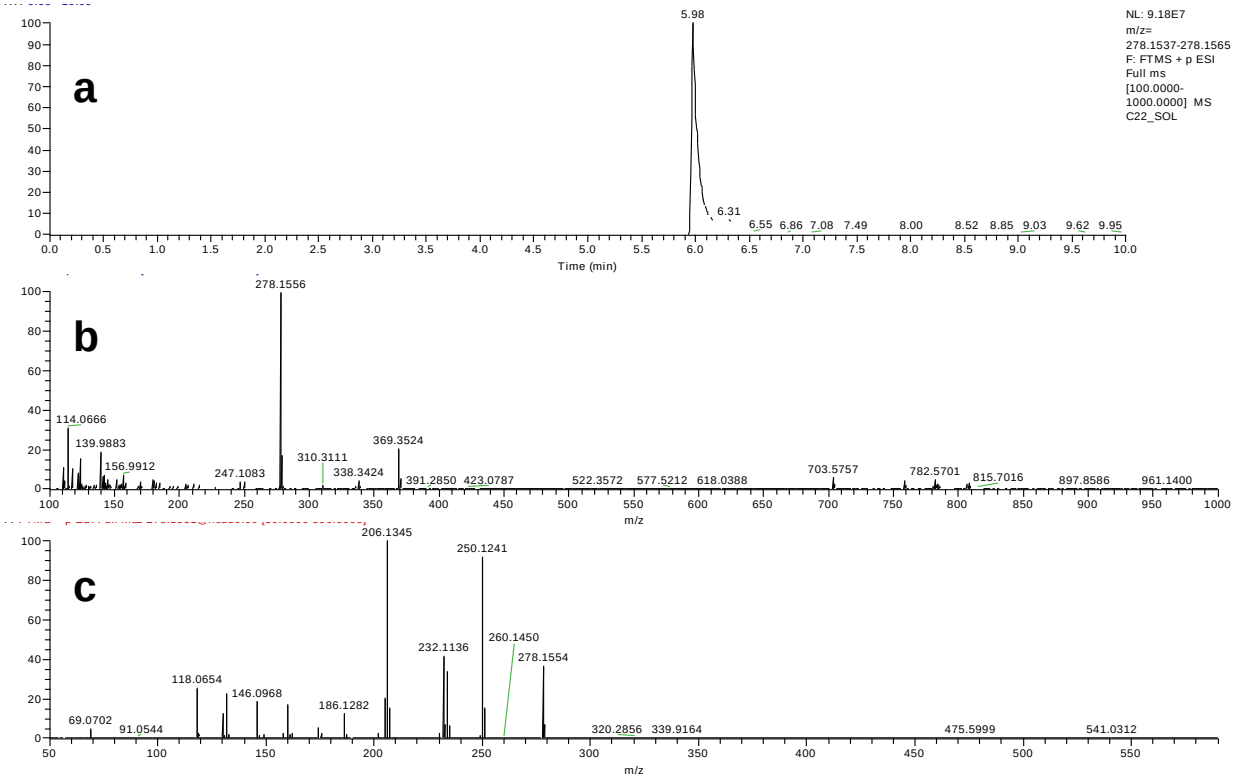
S1.28 LC-HRMS (a), full scan MS spectrum (b) and MS^2 at NCE 25 (c) of synthesised PB-22 ethyl ester, compound 15



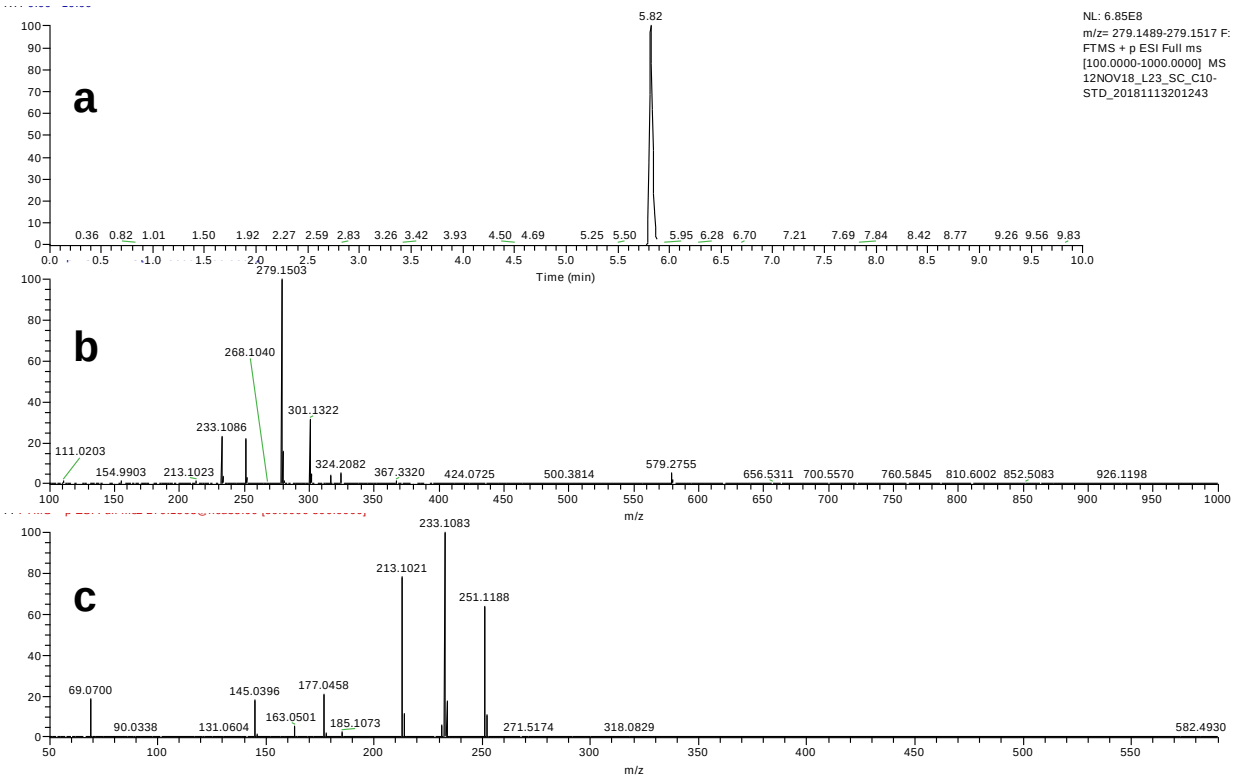
S1.29 LC-HRMS (a), full scan MS spectrum (b) and MS^2 at NCE 30 (c) of synthesised NPB-22 ethyl ester, compound 11



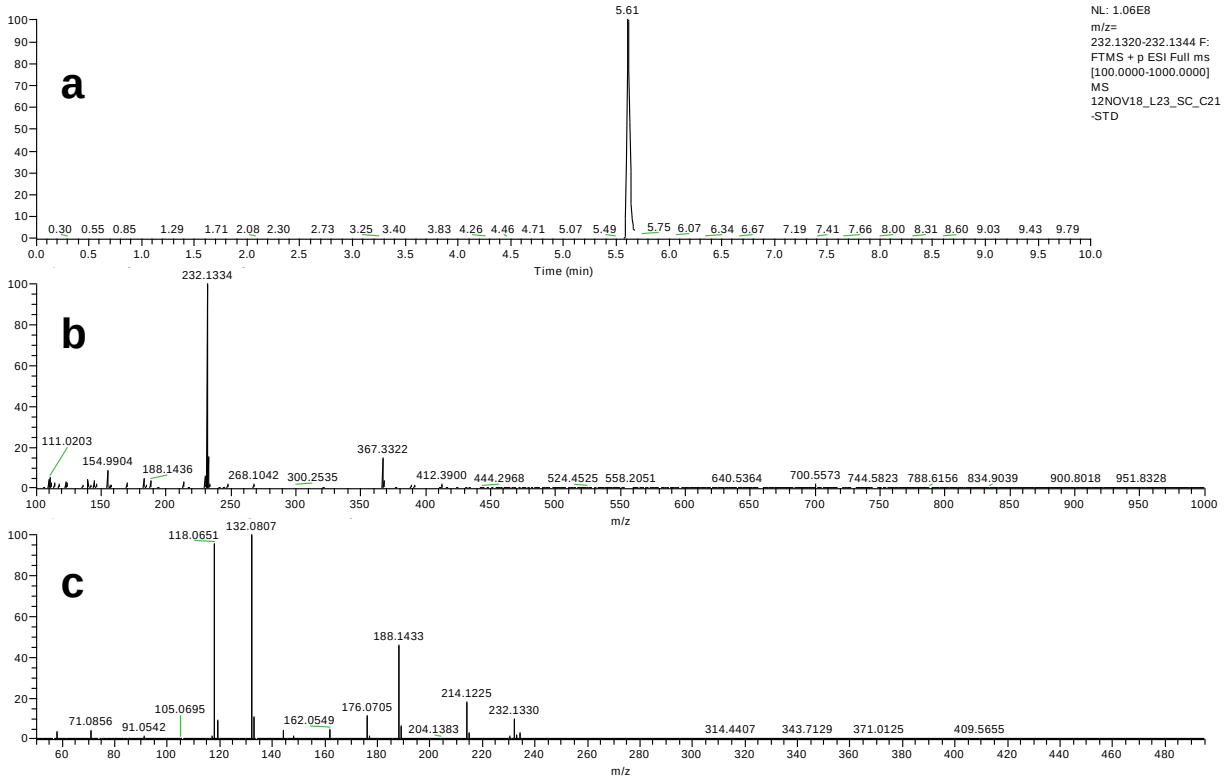
S1.30 LC-HRMS (a), full scan MS spectrum (b) and MS^2 at NCE 30 (c) of synthesised 5F-PB-22 ethyl ester, compound 14



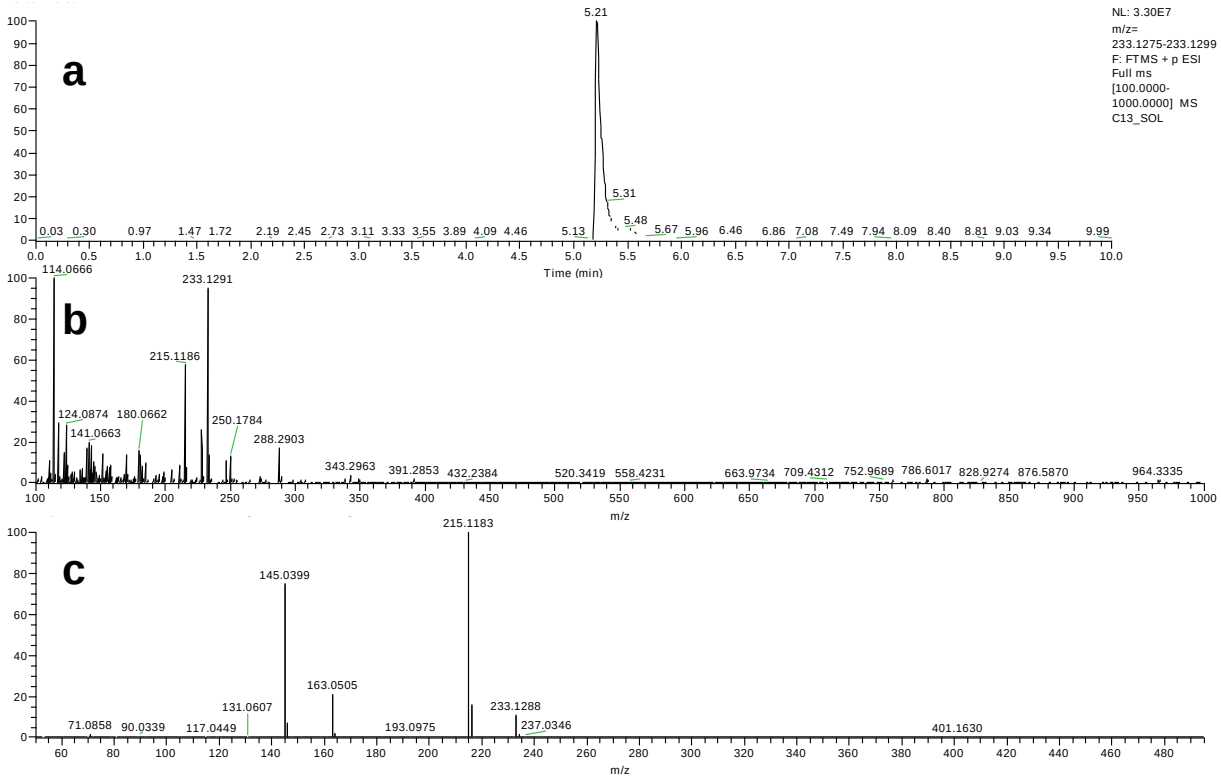
S1.31 LC-HRMS (a), full scan MS spectrum (b) and MS^2 at NCE 35 (c) of synthesised 5F-NPB-22 ethyl ester, compound 10



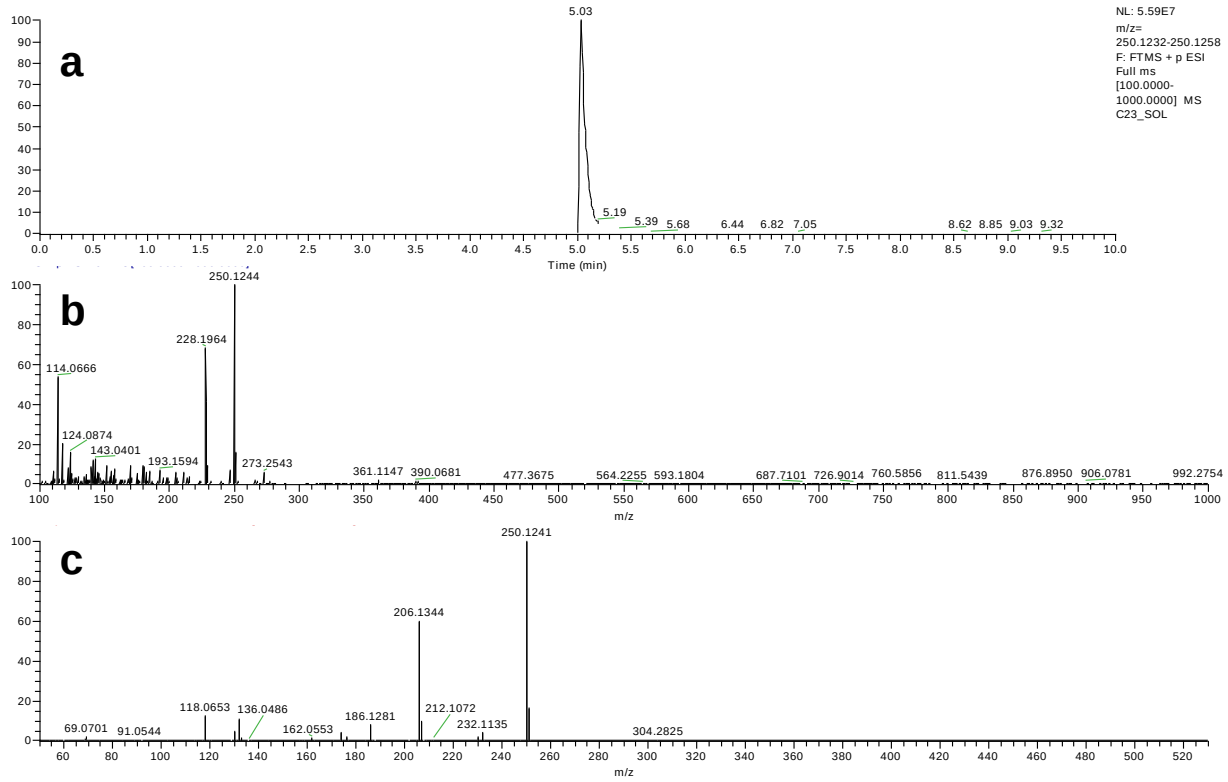
S1.32 LC-HRMS (a), full scan MS spectrum (b) and MS^2 at NCE 40 (c) of synthesised PB-22 COOH, compound 17



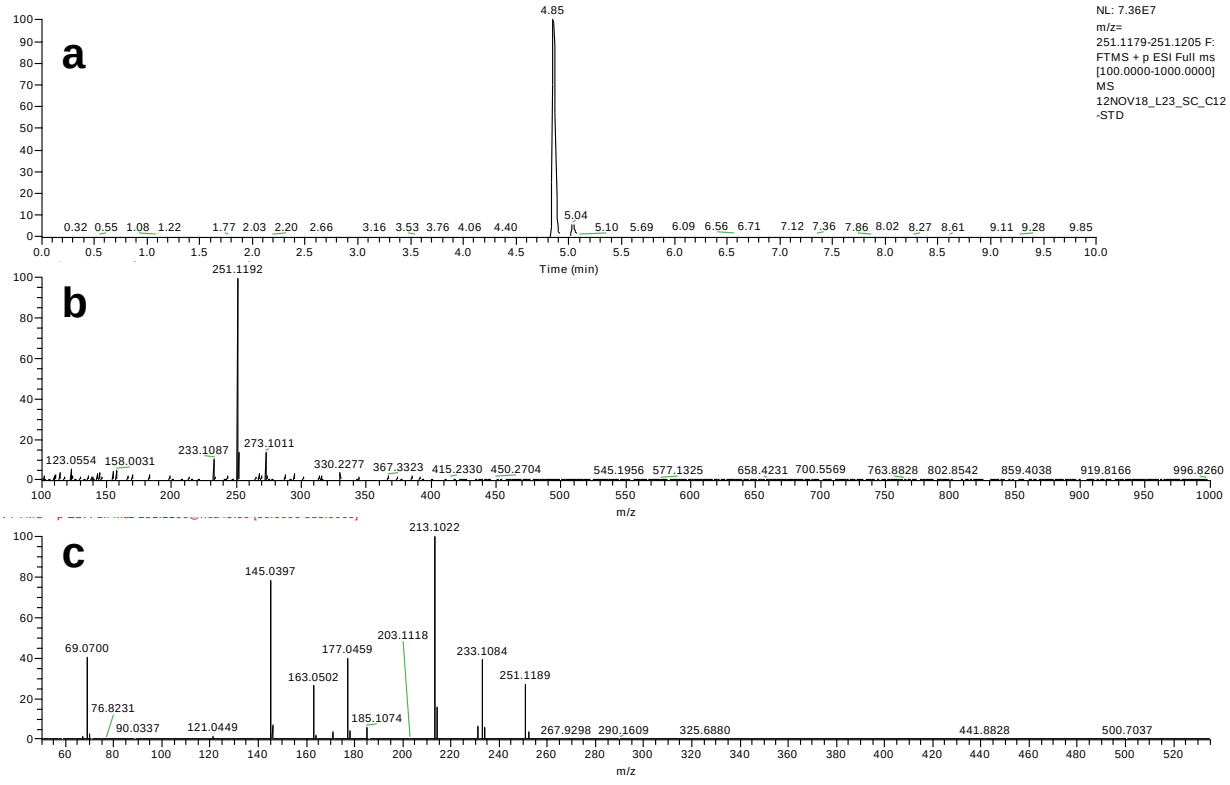
S1.33 LC-HRMS (a), full scan MS spectrum (b) and MS^2 at NCE 45 (c) of synthesised NPB-22-COOH, compound 13



S1.34 LC-HRMS (a), full scan MS spectrum (b) and MS^2 at NCE 20 (c) of synthesised 5F-PB-22-COOH, compound 16

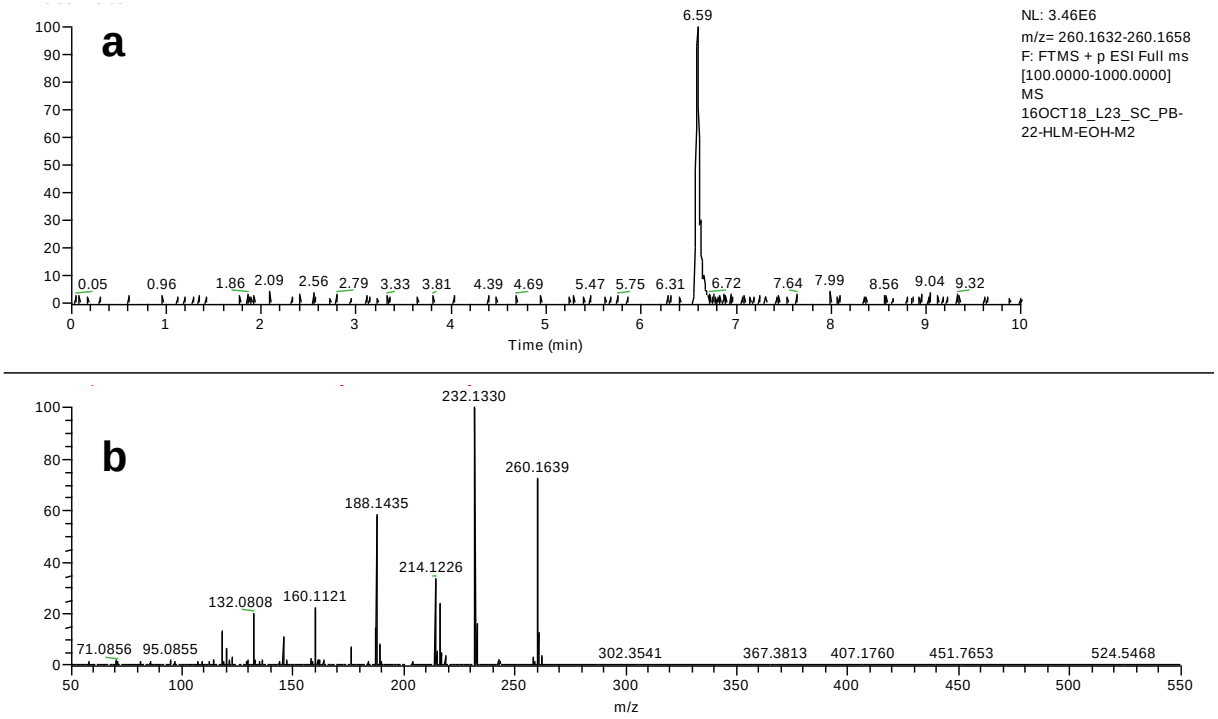


S1.35 LC-HRMS (a), full scan MS spectrum (b) and MS^2 at NCE 45 (c) of synthesised 5F-NPB-22-COOH, compound 12

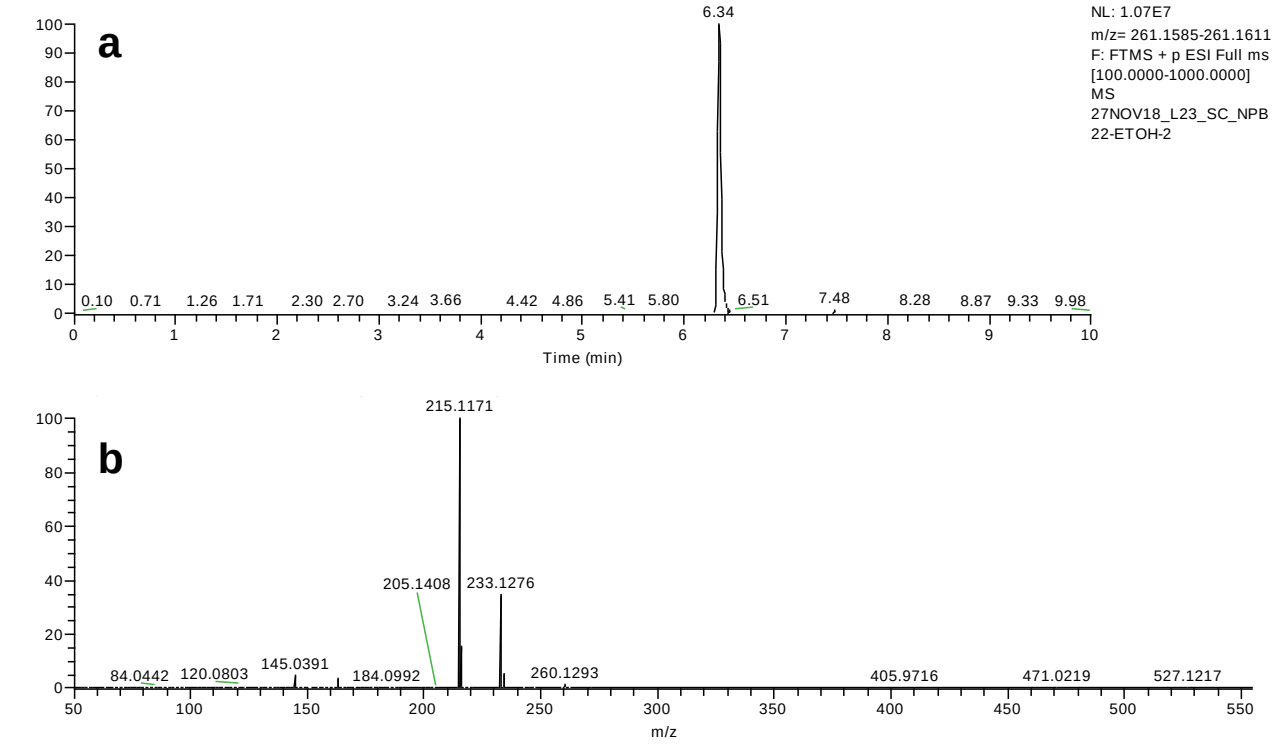


S2: Full MS and MS^2 results of ethyl ester and ester hydrolysis of PB-22, NPB-22, 5F-PB-22, and 5F-NPB-22 incubations with HLM

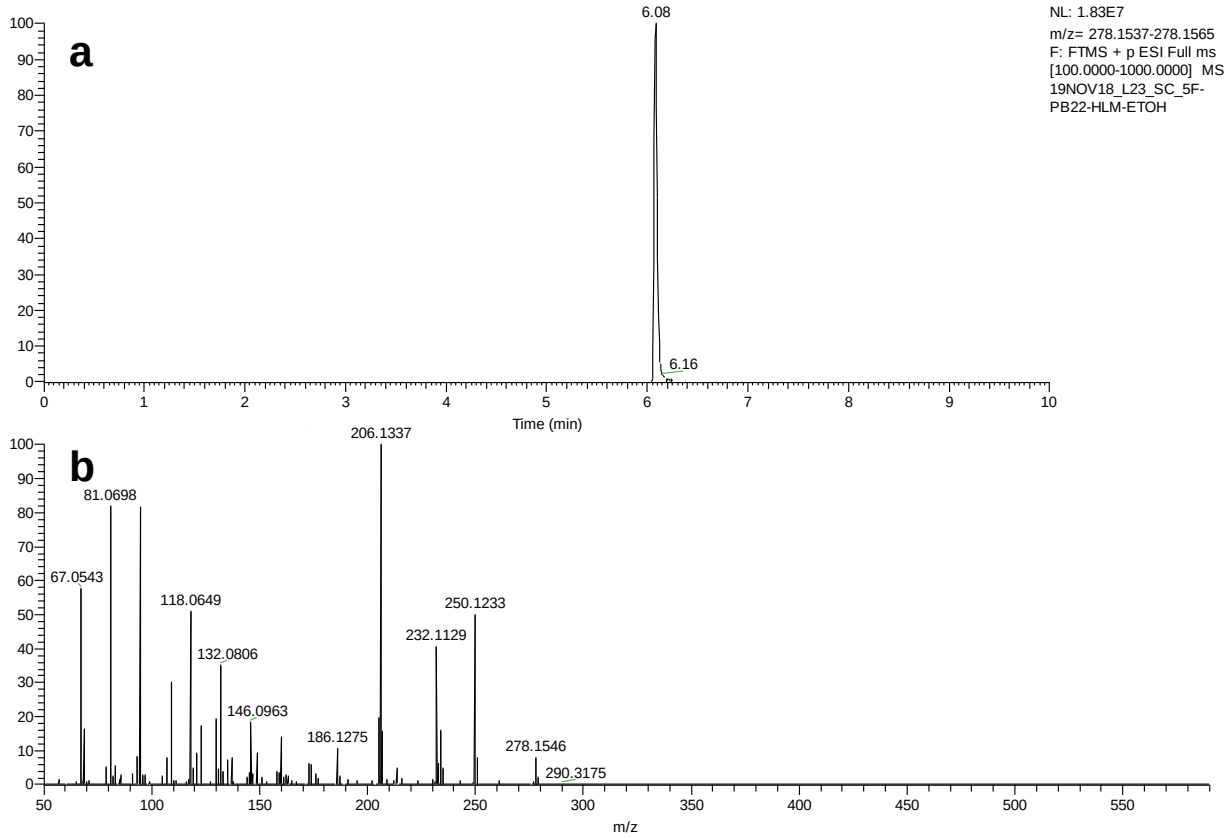
S2.1 PB-22 ethyl ester detection/confirmation: full scan MS EIC (a) and MS^2 at NCE 25 (b)



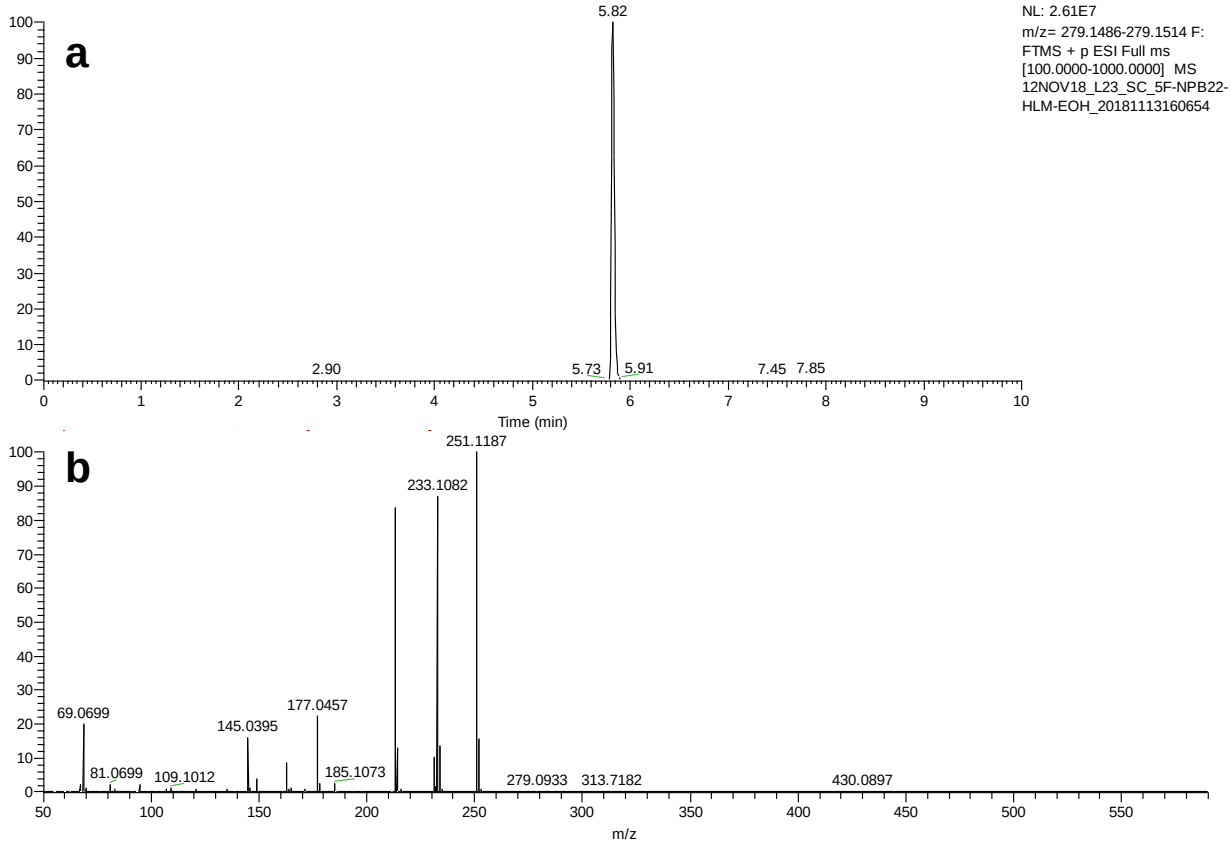
S2.2 NPB-22 ethyl ester detection/confirmation: full scan MS EIC (a) and MS^2 at NCE 30 (b)



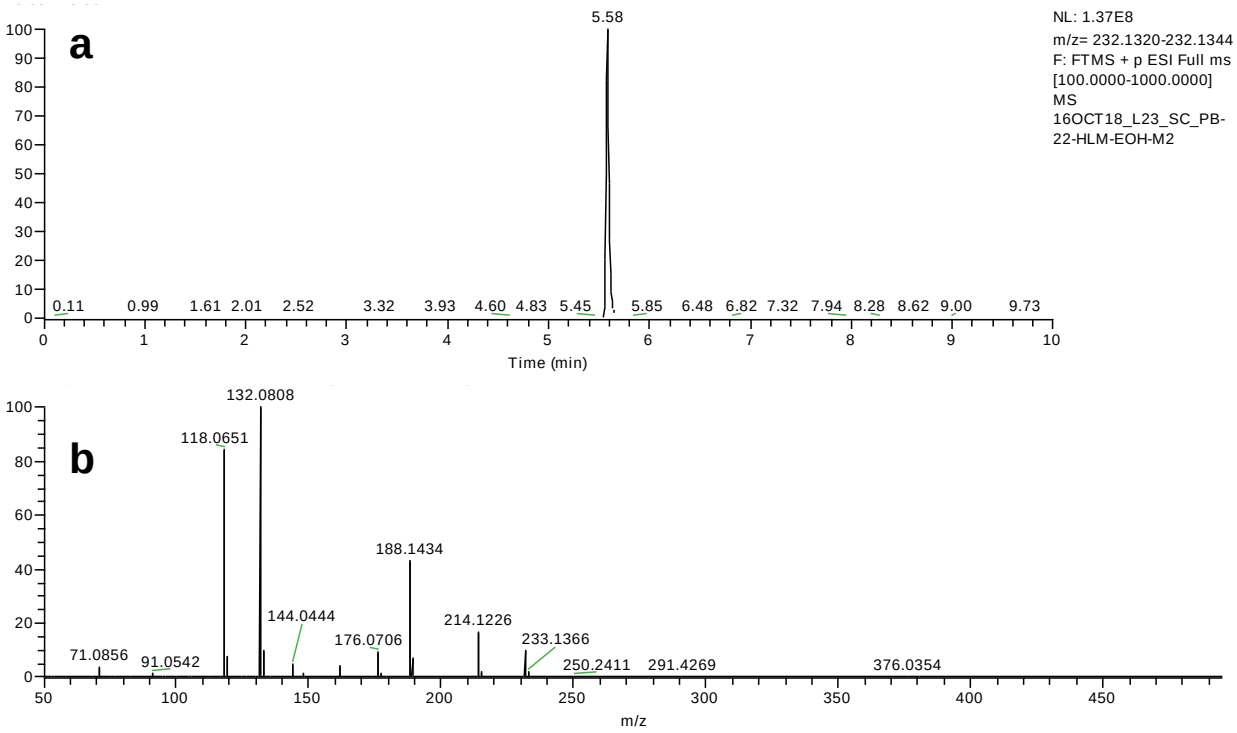
S2.3 5F-PB-22 ethyl ester detection/confirmation: full scan MS EIC (a) and MS^2 at NCE 30 (b)



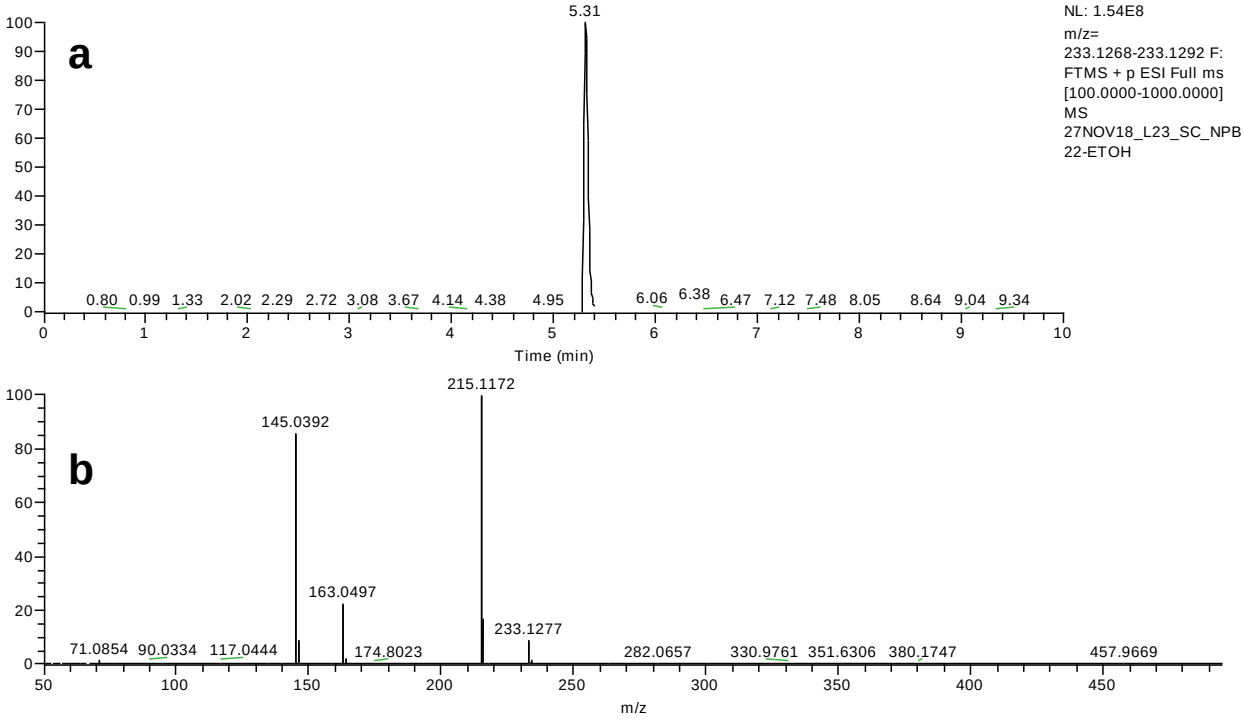
S2.4 5F-NPB-22 ethyl ester detection/confirmation: full scan MS EIC (a) and MS^2 at NCE 35 (b)



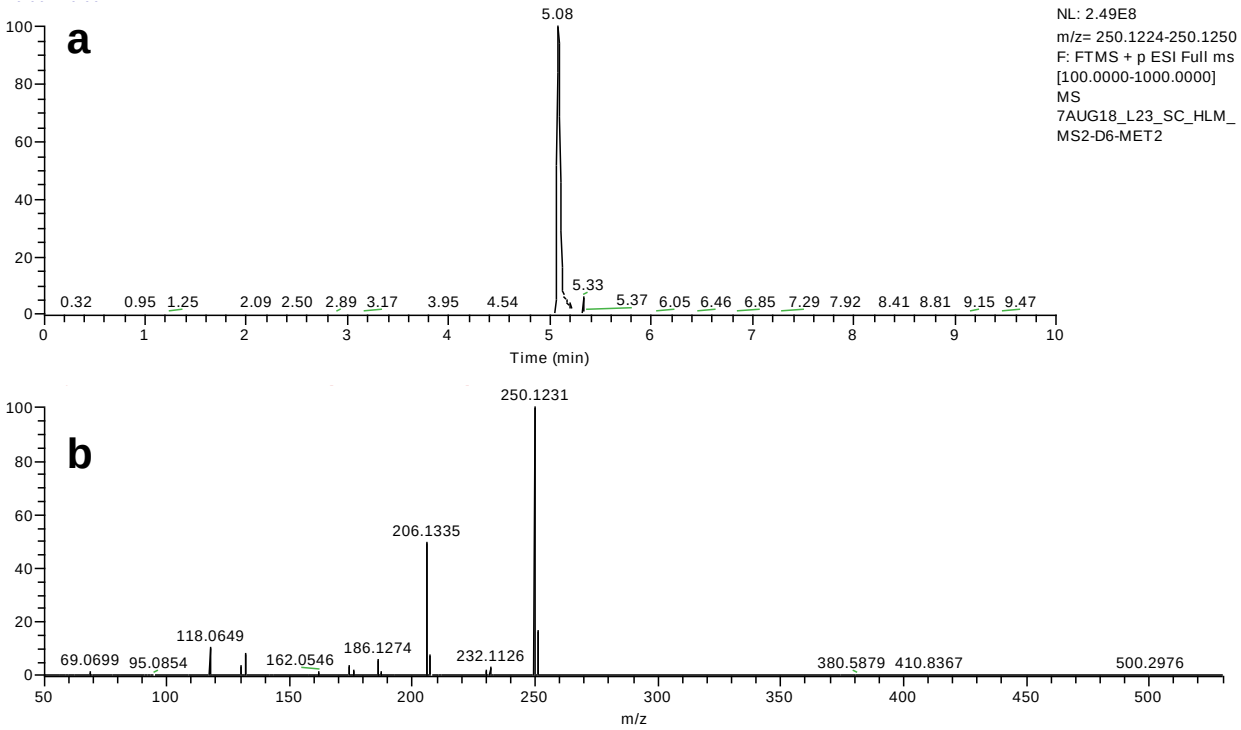
S2.5 PB-22 ester hydrolysis detection/confirmation: full scan MS EIC (a) and MS^2 at NCE40 (b)



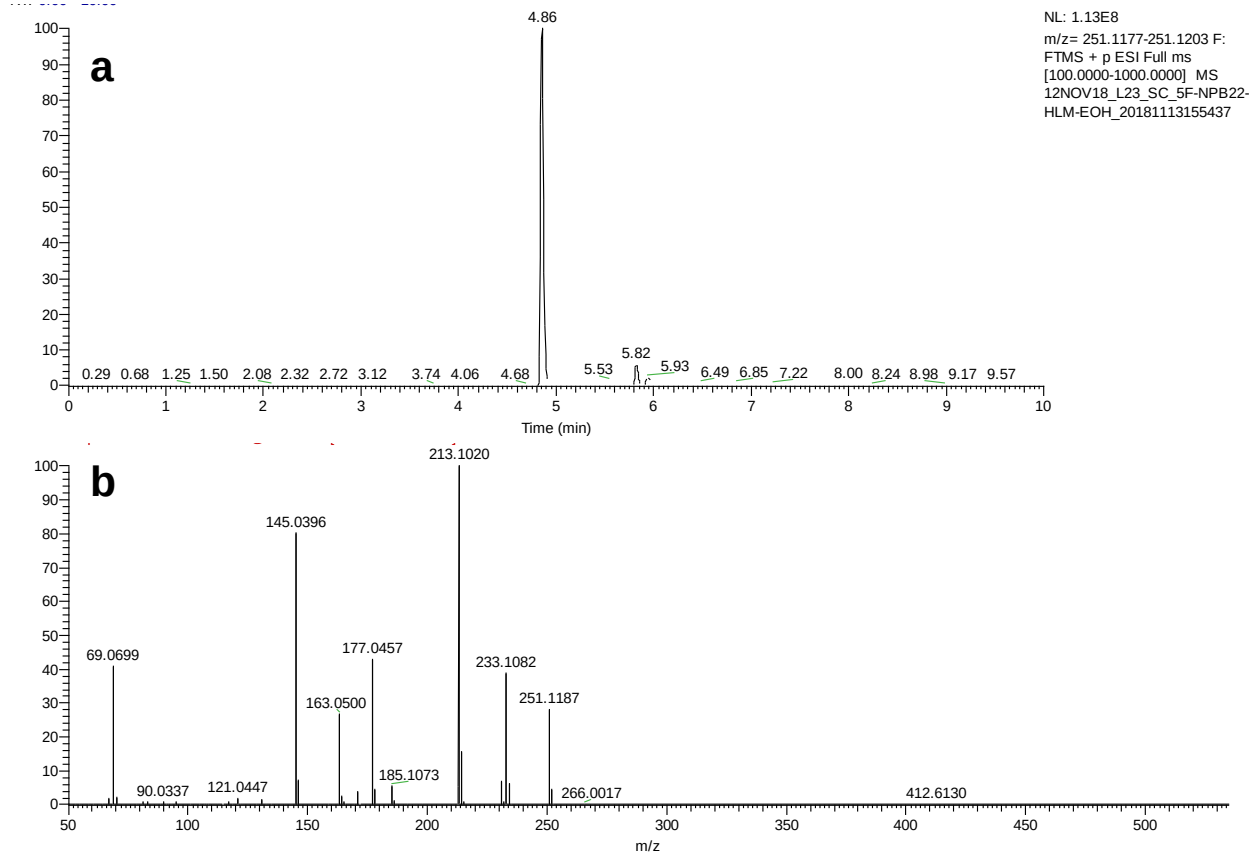
S2.6 NPB-22 ester hydrolysis detection/confirmation: full scan MS EIC (a) and MS^2 at NCE 45 (b)



S2.7 5F-PB-22 ester hydrolysis detection/confirmation: full scan MS EIC (a) and MS^2 at NCE 20 (b)



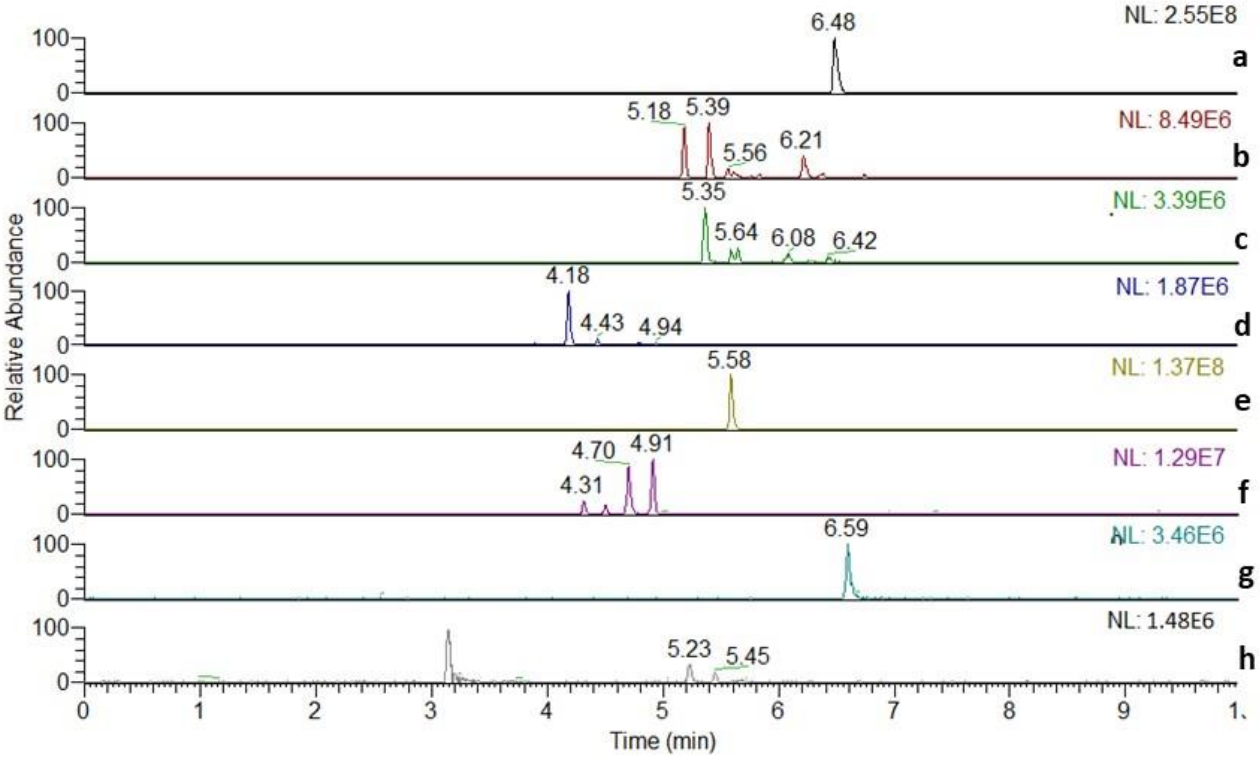
S2.8 5F-NPB-22 ester hydrolysis detection/confirmation: full scan MS EIC (a) and MS^2 at NCE 45 (b)



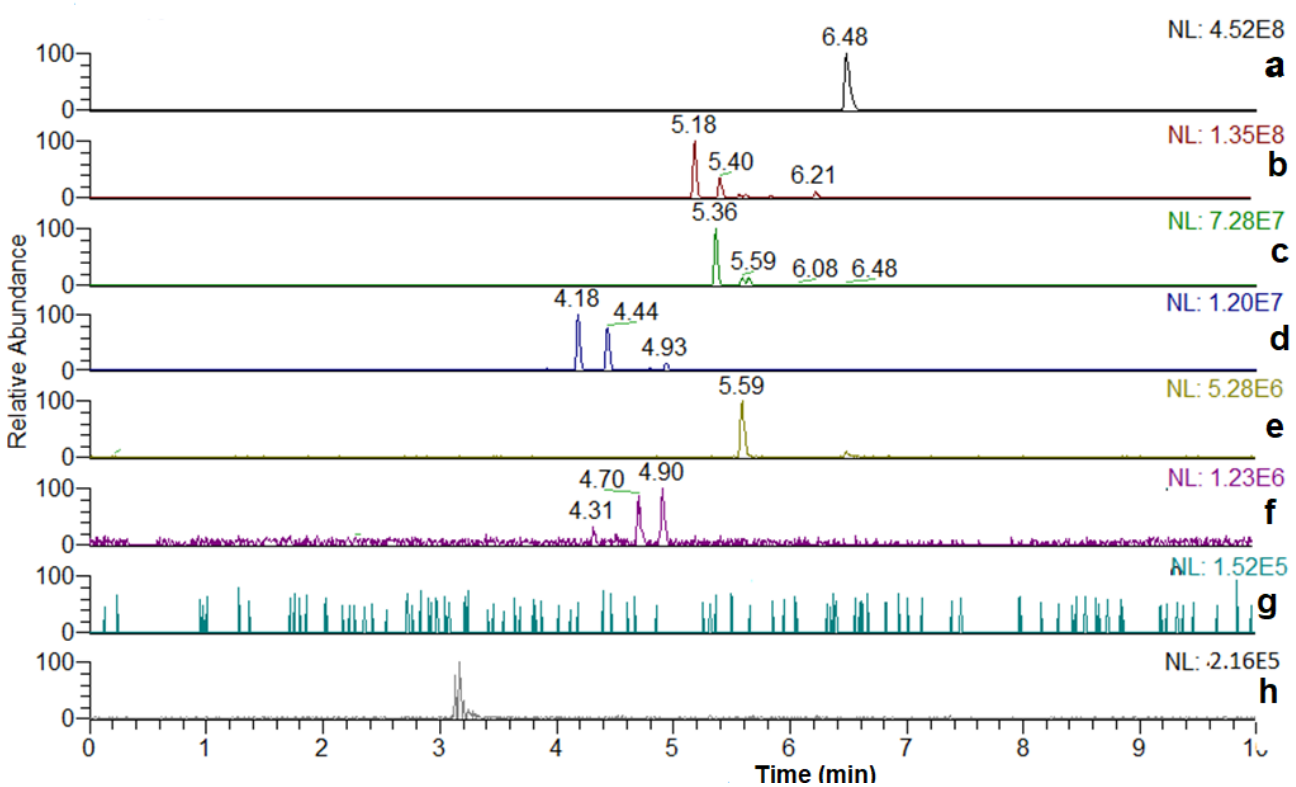
S3: Overlaid extracted ion chromatograms (exact mass ± 2.5 ppm) of the major detected metabolites in the presence of ethanol, ethanol and inhibitor, and negative control (without HLM).

Overlaid extracted ion chromatograms (exact mass ± 2.5 ppm) of the major detected PB-22 HLM-derived metabolites (**S3.1-S3.4**) in the presence of ethanol, ethanol + inhibitors and negative control: a = parent drug PB-22; b = monohydroxylation; c = carbonylation; d = monohydroxylation plus N-dealkylation; e = ester hydrolysis (**17**); f = ester hydrolysis plus monohydroxylation; g = PB-22 ethyl ester (**15**); PB-22 ethyl ester plus monohydroxylation

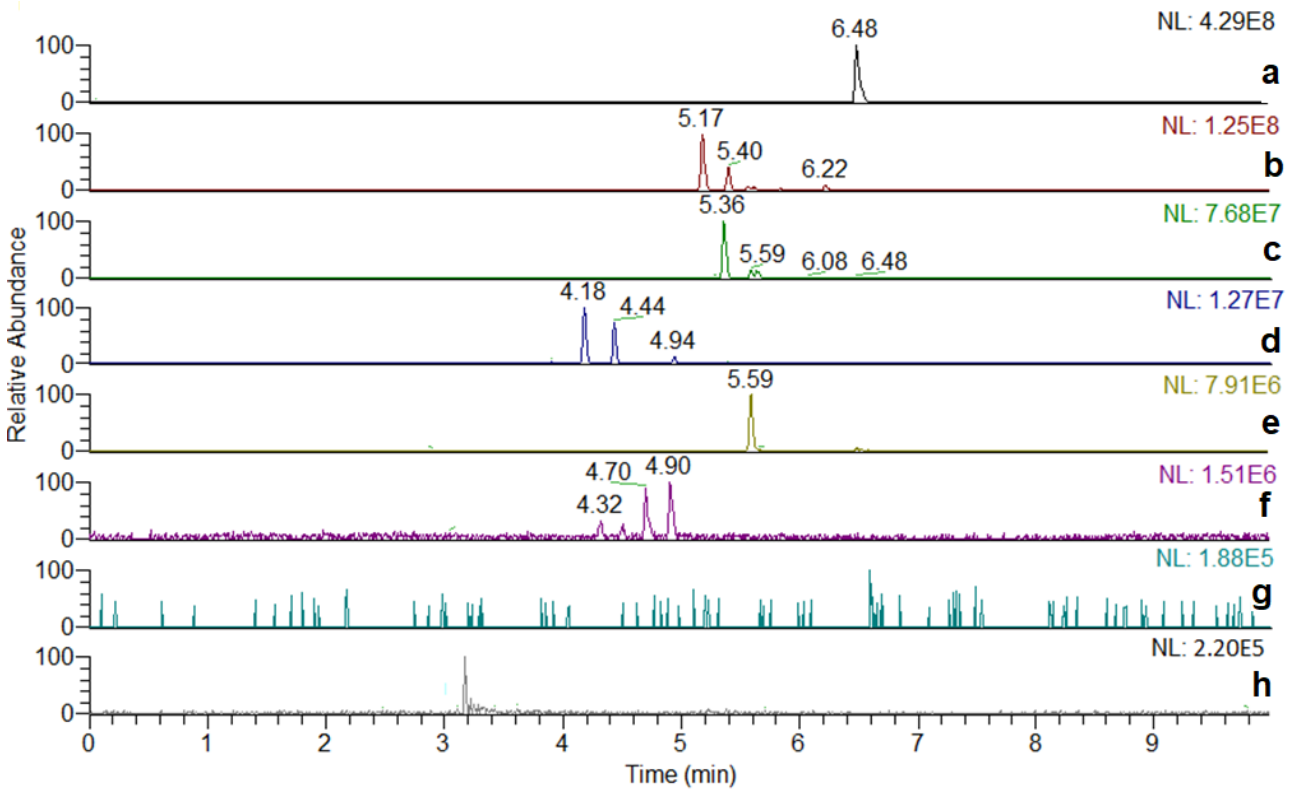
S3.1 PB-22 HLM-derived metabolites in the presence of ethanol



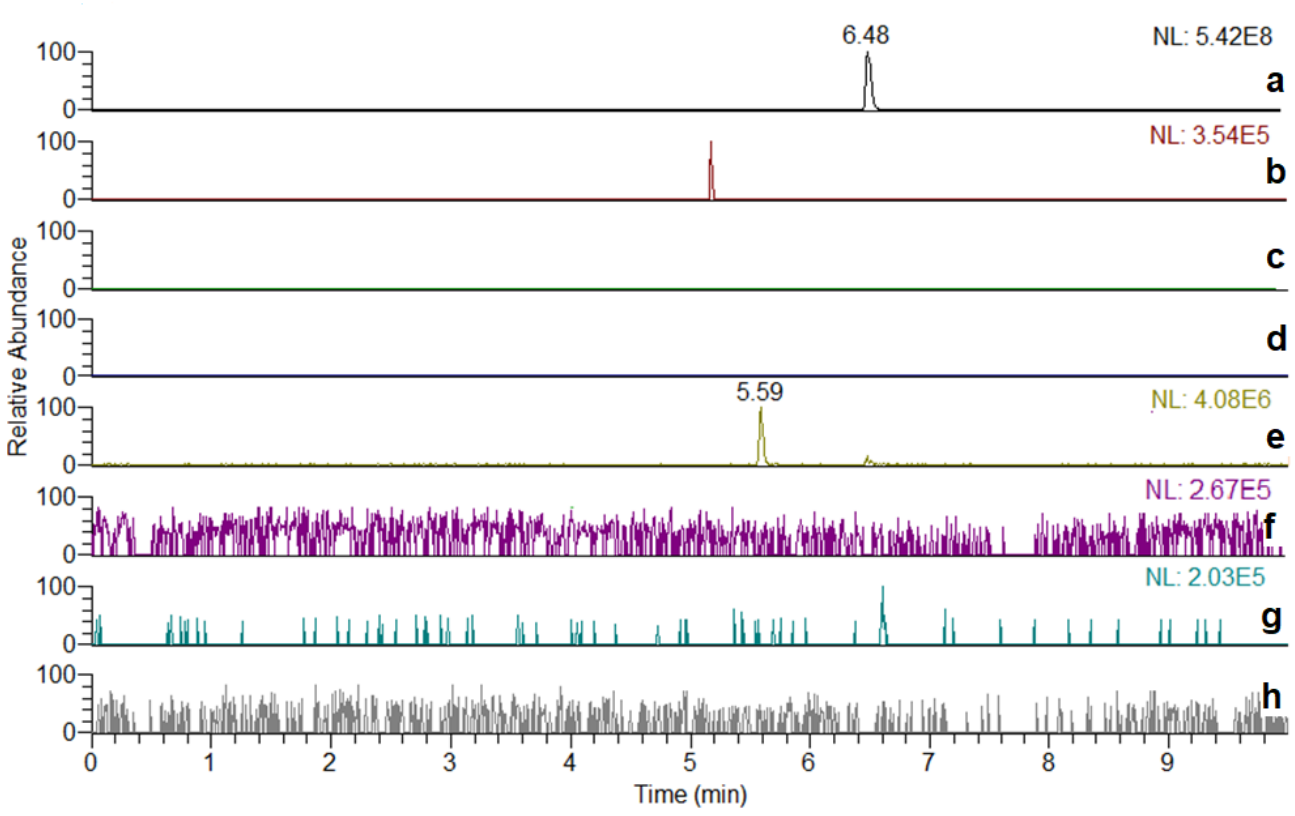
S3.2 PB-22 HLM-derived metabolites in the presence of ethanol and BNPP



S3.3 PB-22 HLM-derived metabolites in the presence of ethanol and NaF

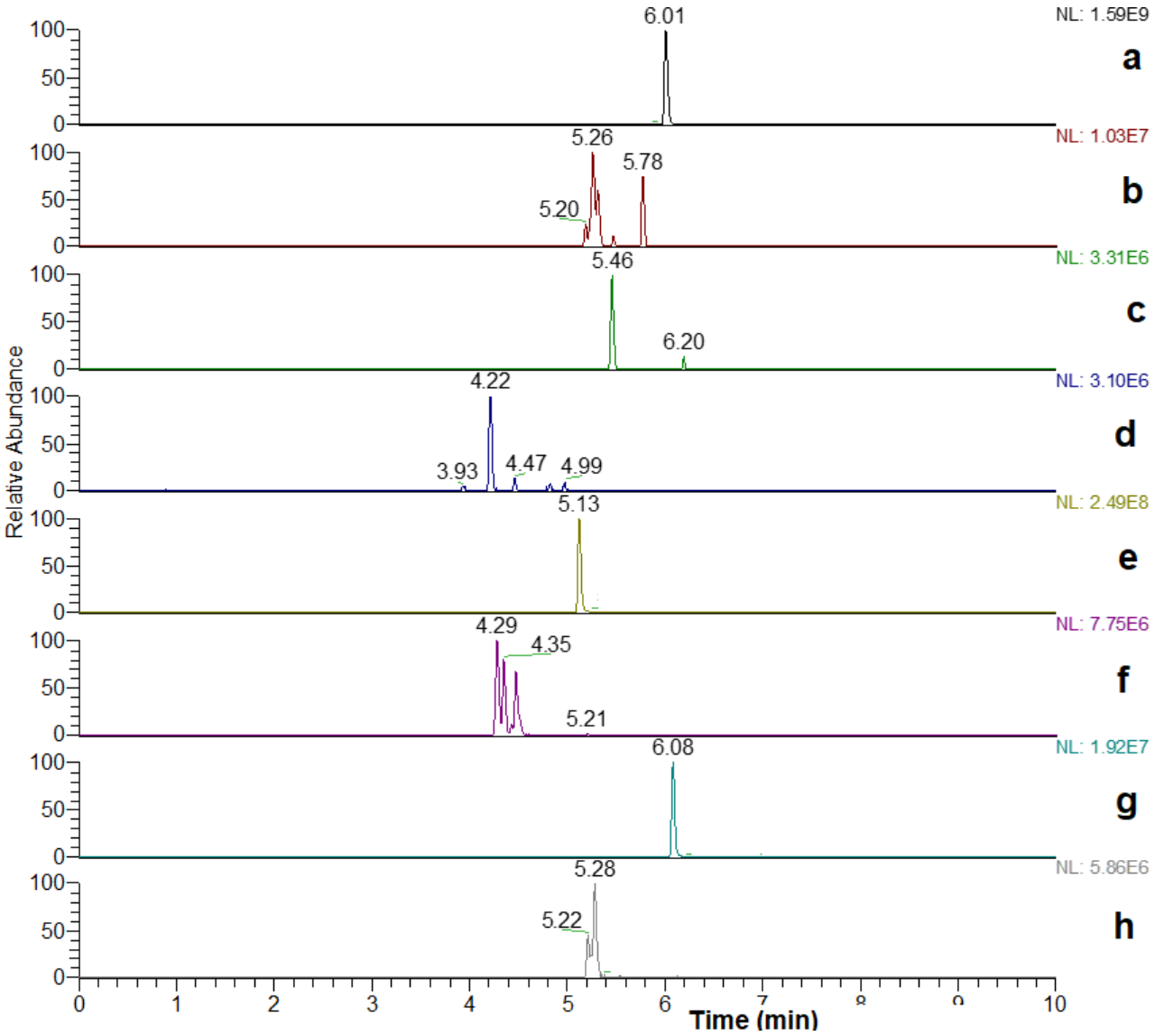


S3.4 Negative control PB-22 incubated with buffer in the presence of ethanol

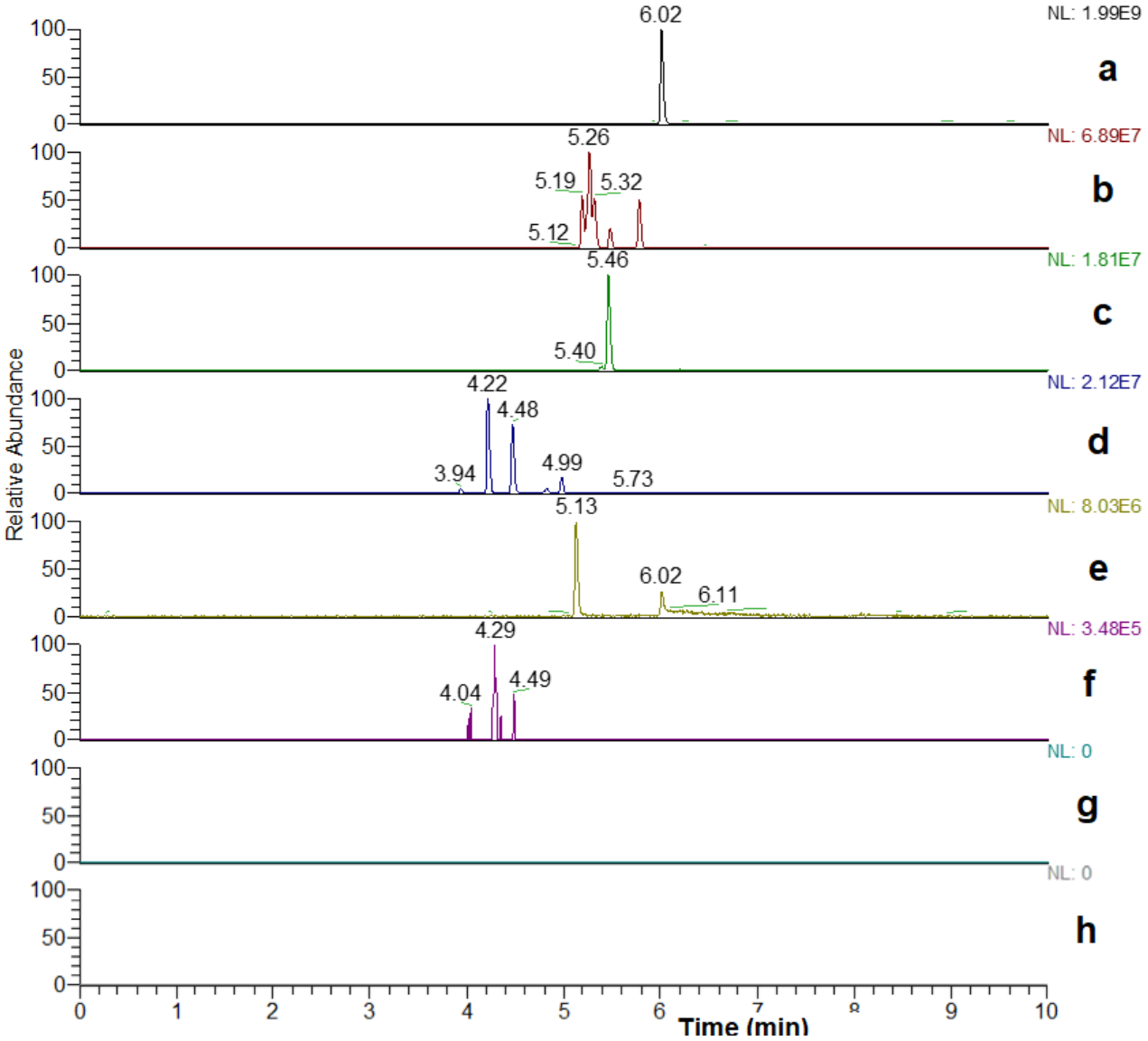


Overlaid extracted ion chromatograms (exact mass ± 2.5 ppm) of the major detected 5F-PB-22 HLM-derived metabolites (**S3.5-S3.8**) in the presence of ethanol, ethanol + inhibitor and negative control: a = parent drug 5F-PB-22; b = monohydroxylation; c = carbonylation; d = monohydroxylation plus N-dealkylation; e = ester hydrolysis (**16**); f = ester hydrolysis plus monohydroxylation; g = 5F-PB-22 ethyl ester (**14**); h = 5F-PB-22 ethyl ester plus monohydroxylation

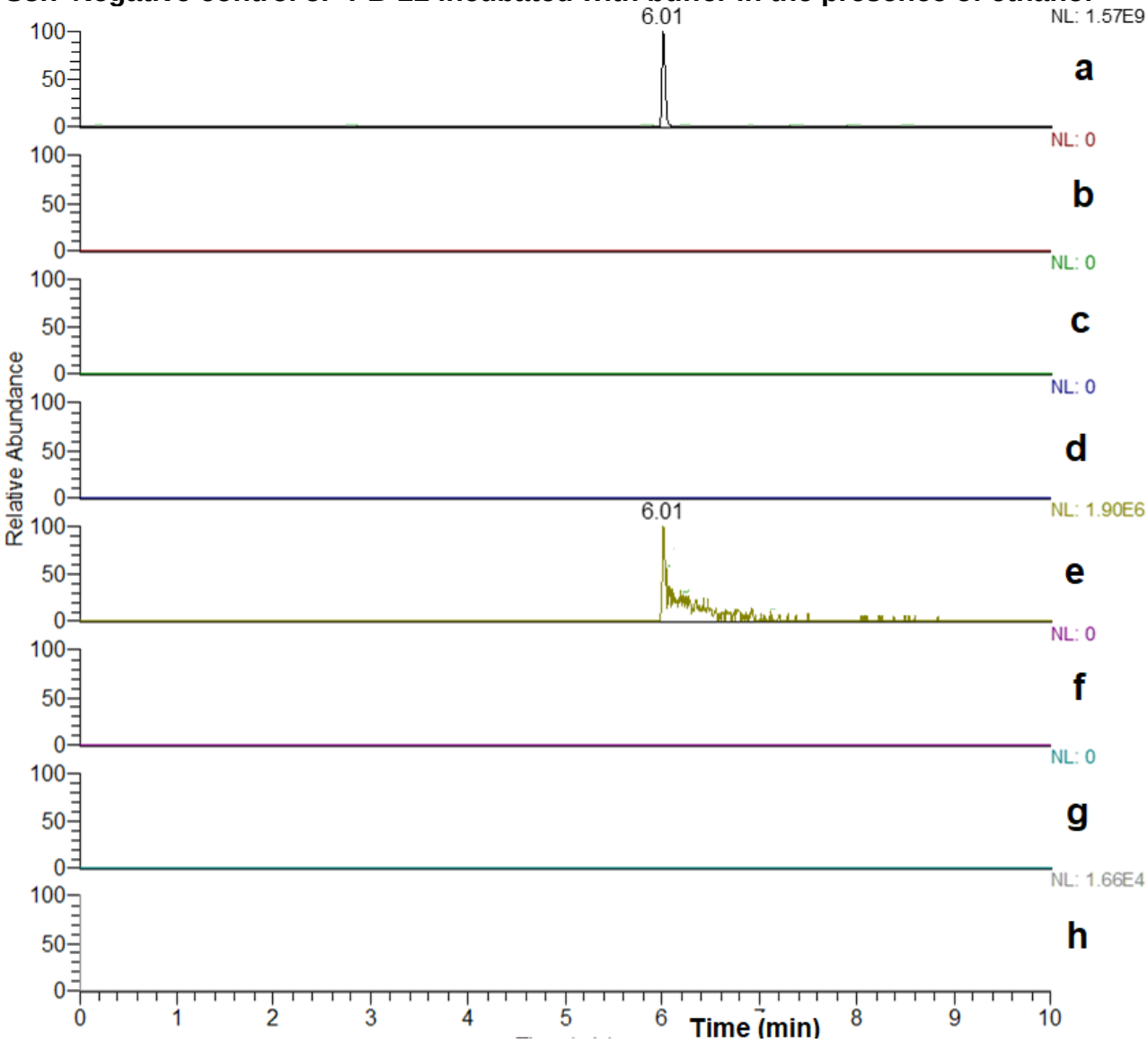
S3.5 5F-PB-22 HLM-derived metabolites in the presence of ethanol



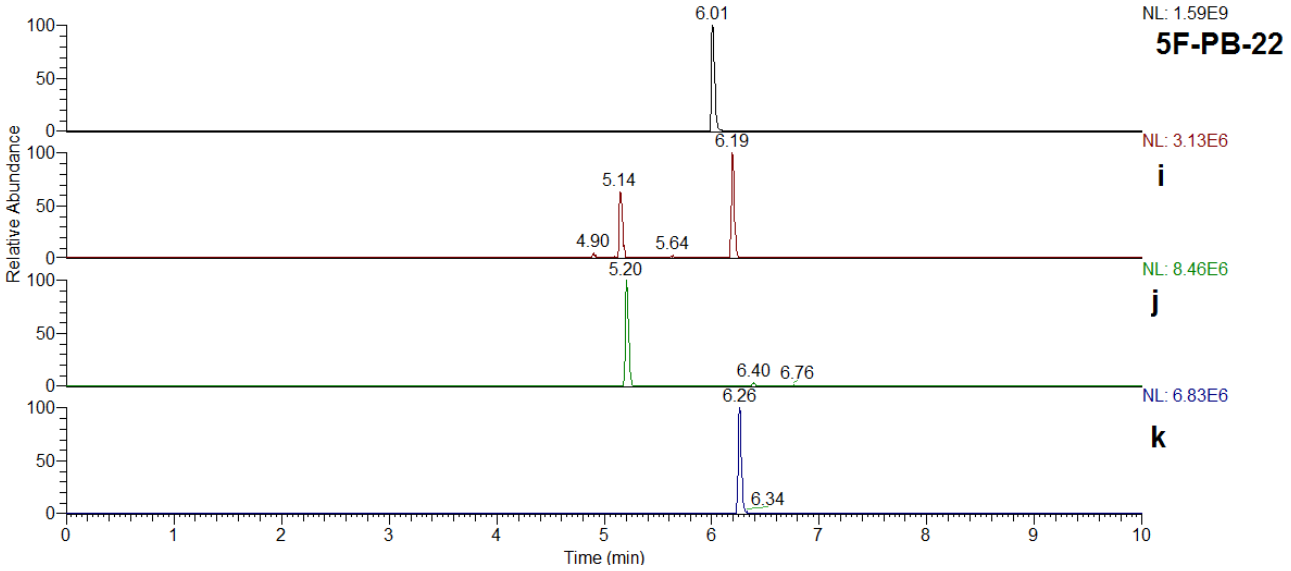
S3.6 5F-PB-22 HLM-derived metabolites in the presence of ethanol and BNPP



S3.7 Negative control 5F-PB-22 incubated with buffer in the presence of ethanol

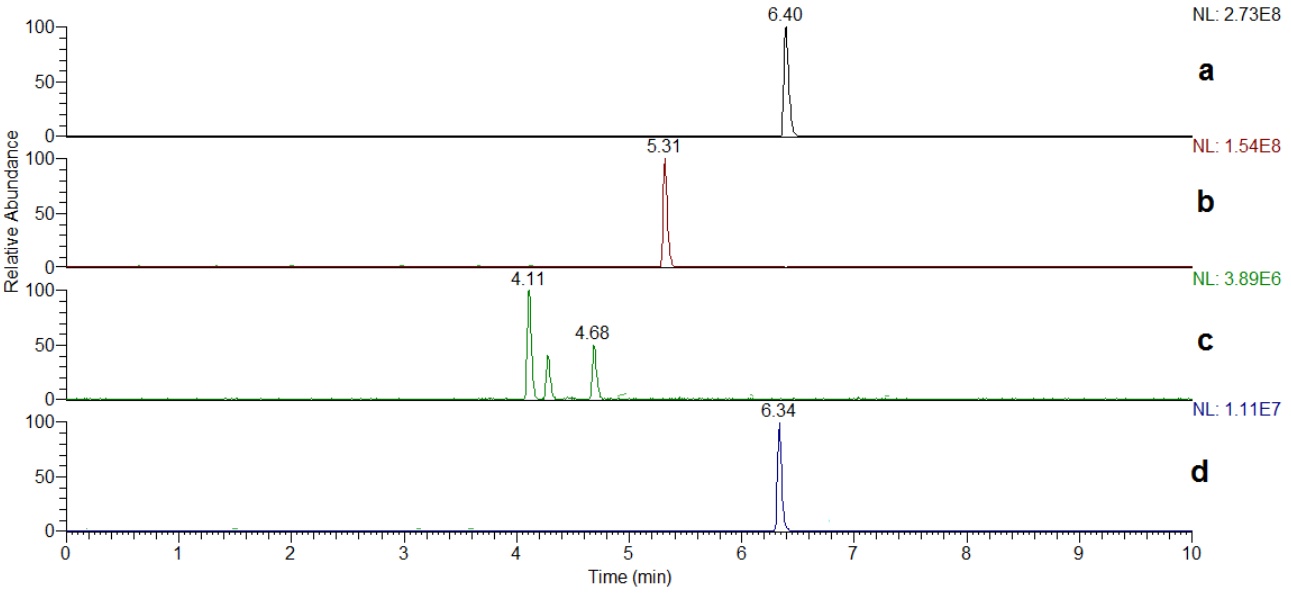


S3.8 5F-PB-22 HLM-derived metabolites, i = oxidative defluorination to COOH, j = oxidative defluorination and k = reductive defluorination

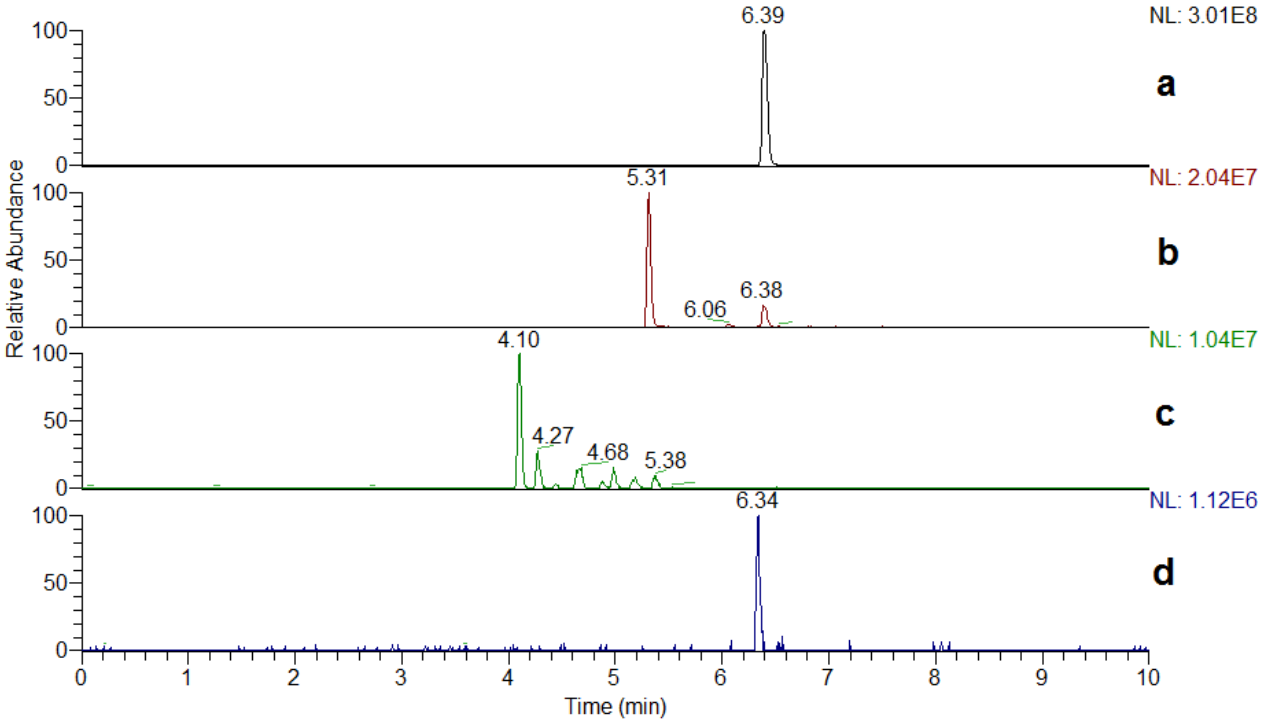


Overlaid extracted ion chromatograms (exact mass ± 2.5 ppm) of the major detected NPB-22 HLM-derived metabolites (S3.9-S3.11) in the presence of ethanol, ethanol + inhibitor and negative control: a = parent drug NPB-22; b = ester hydrolysis (**13**); c = ester hydrolysis plus monohydroxylation; d = NPB-22 ethyl ester (**11**)

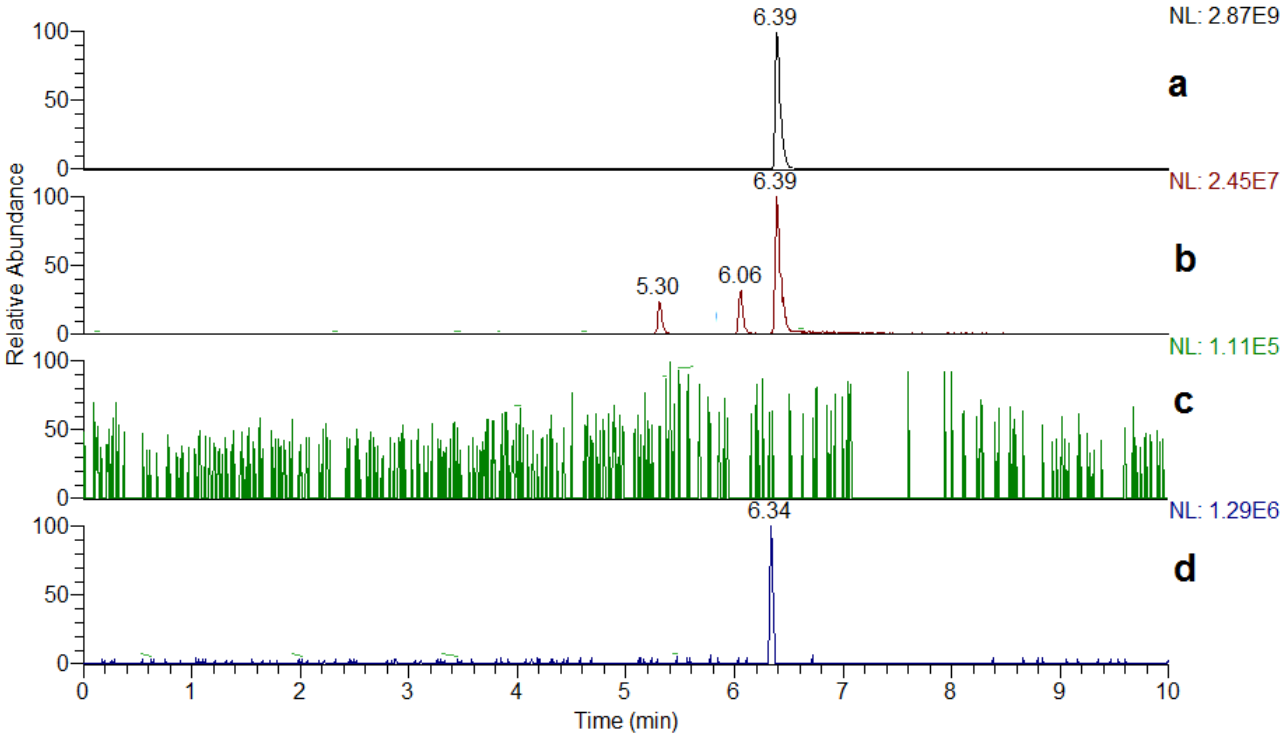
S3.9 NPB-22 HLM-derived metabolites in the presence of ethanol



S3.10 NPB-22 HLM-derived metabolites in the presence of ethanol and BNPP

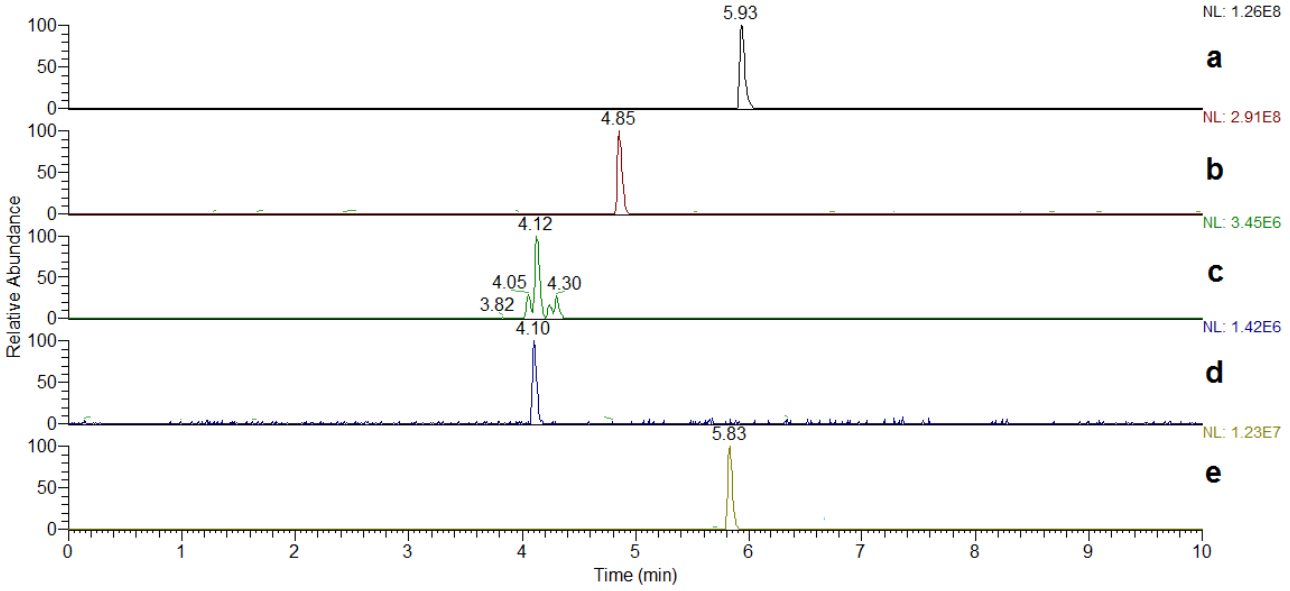


S3.11 Negative control NPB-22 incubated with buffer in the presence of ethanol

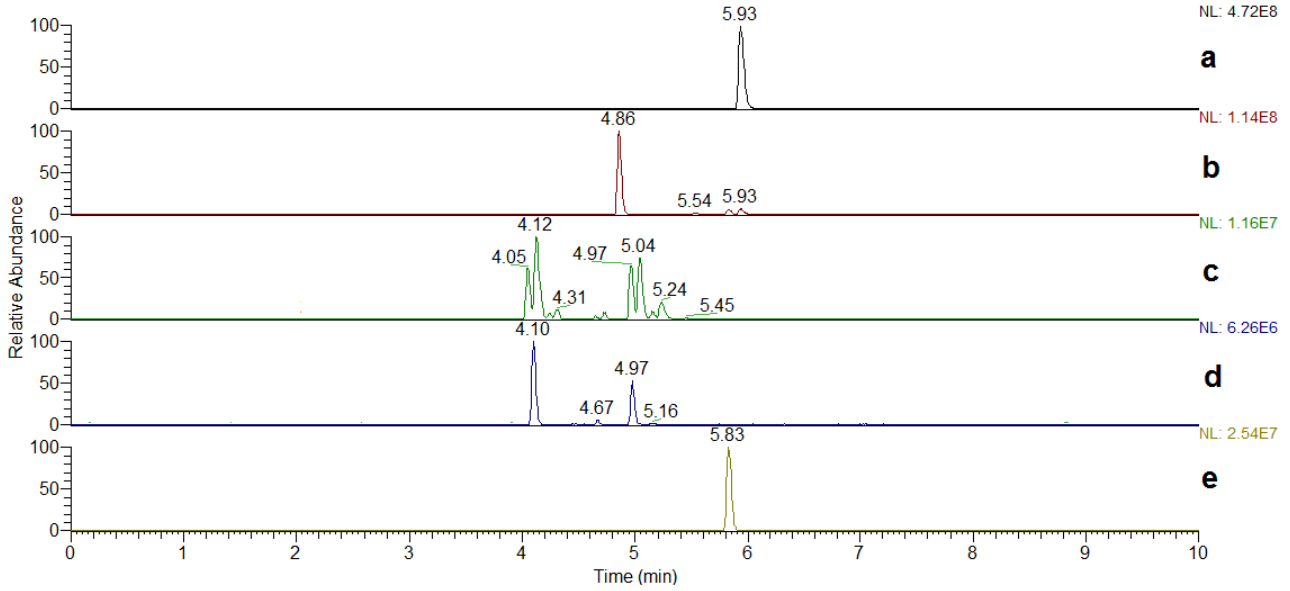


Overlaid extracted ion chromatograms (exact mass ± 2.5 ppm) of the major detected 5F-NPB-22 HLM-derived metabolites (S3.12-S3.14) in the presence of ethanol, ethanol + inhibitor and negative control: a = parent drug 5F-NPB-22; b = ester hydrolysis (**12**); c = ester hydrolysis plus monohydroxylation; d = ester hydrolysis plus oxidative defluorination ; e = 5F-NPB-22 ethyl ester (**10**)

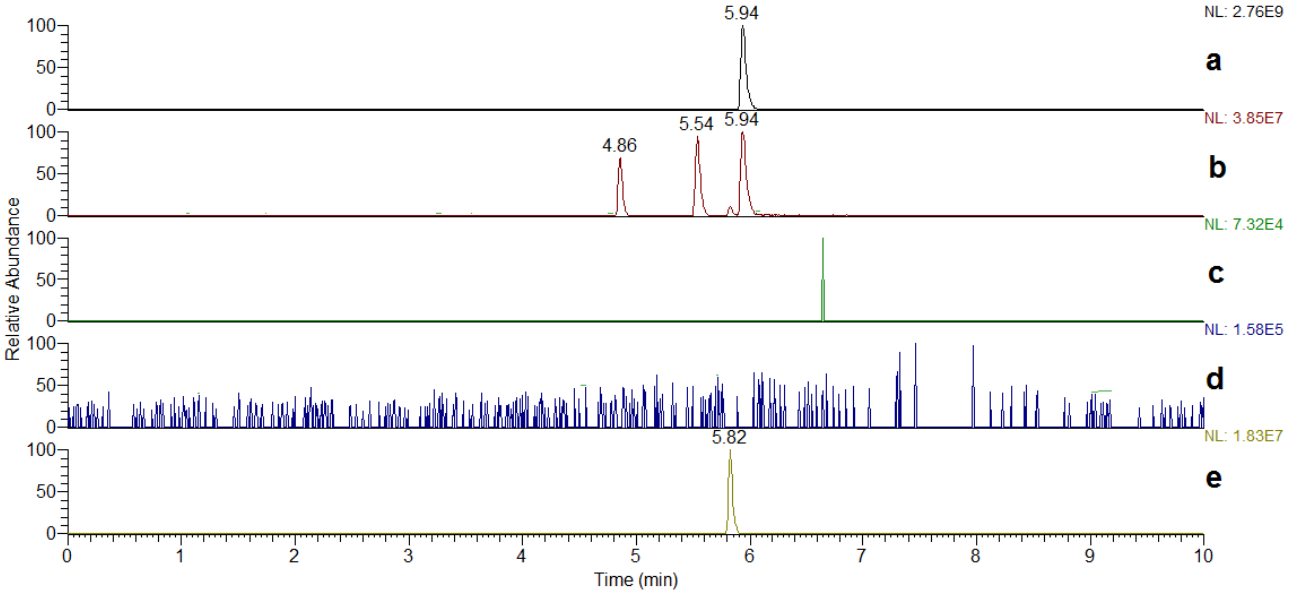
S3.12 5F-NPB-22 HLM-derived metabolites in the presence of ethanol



S3.13 5F-NPB-22 HLM-derived metabolites in the presence of ethanol and BNPP

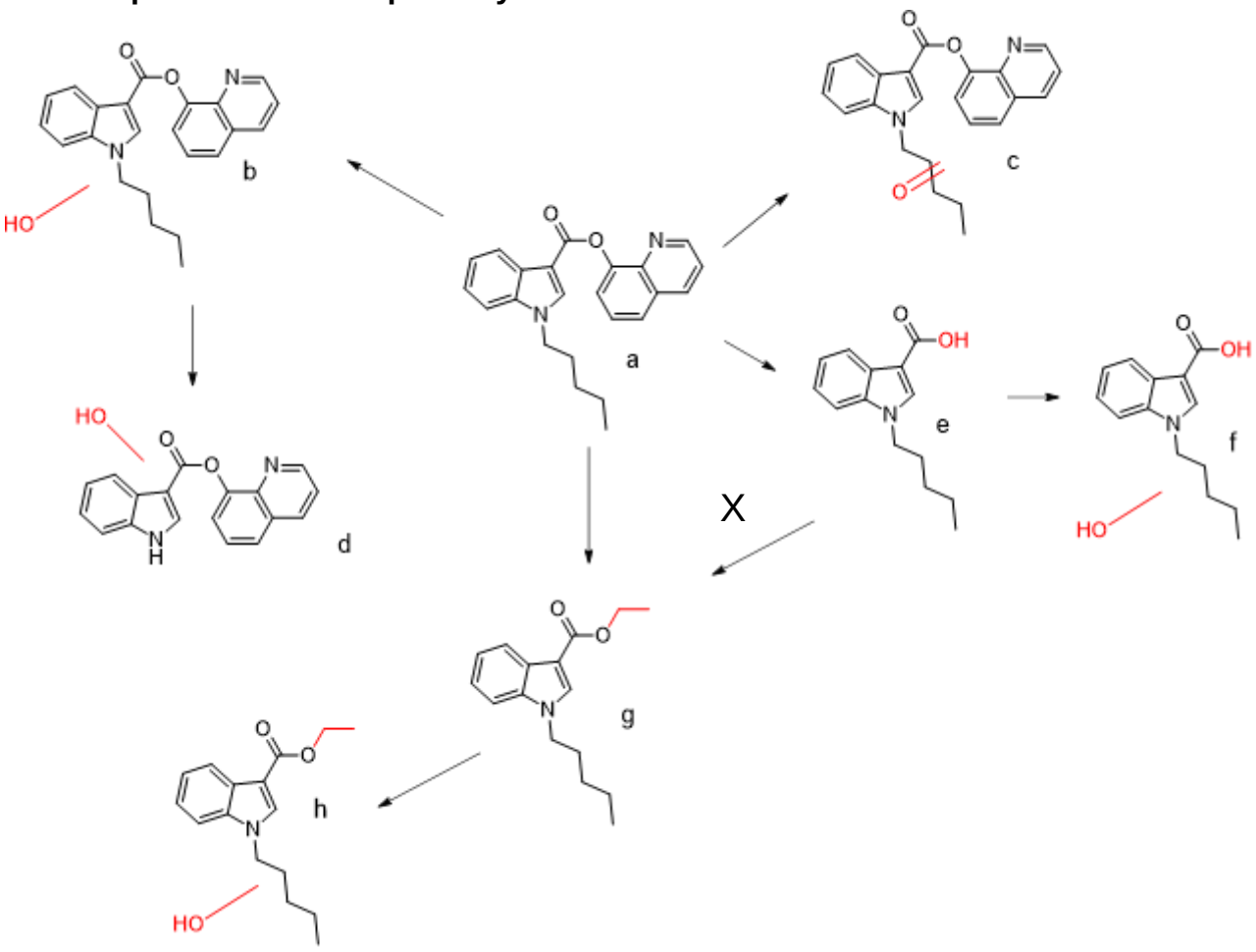


S3.14 Negative control 5F-NPB-22 in the presence of ethanol

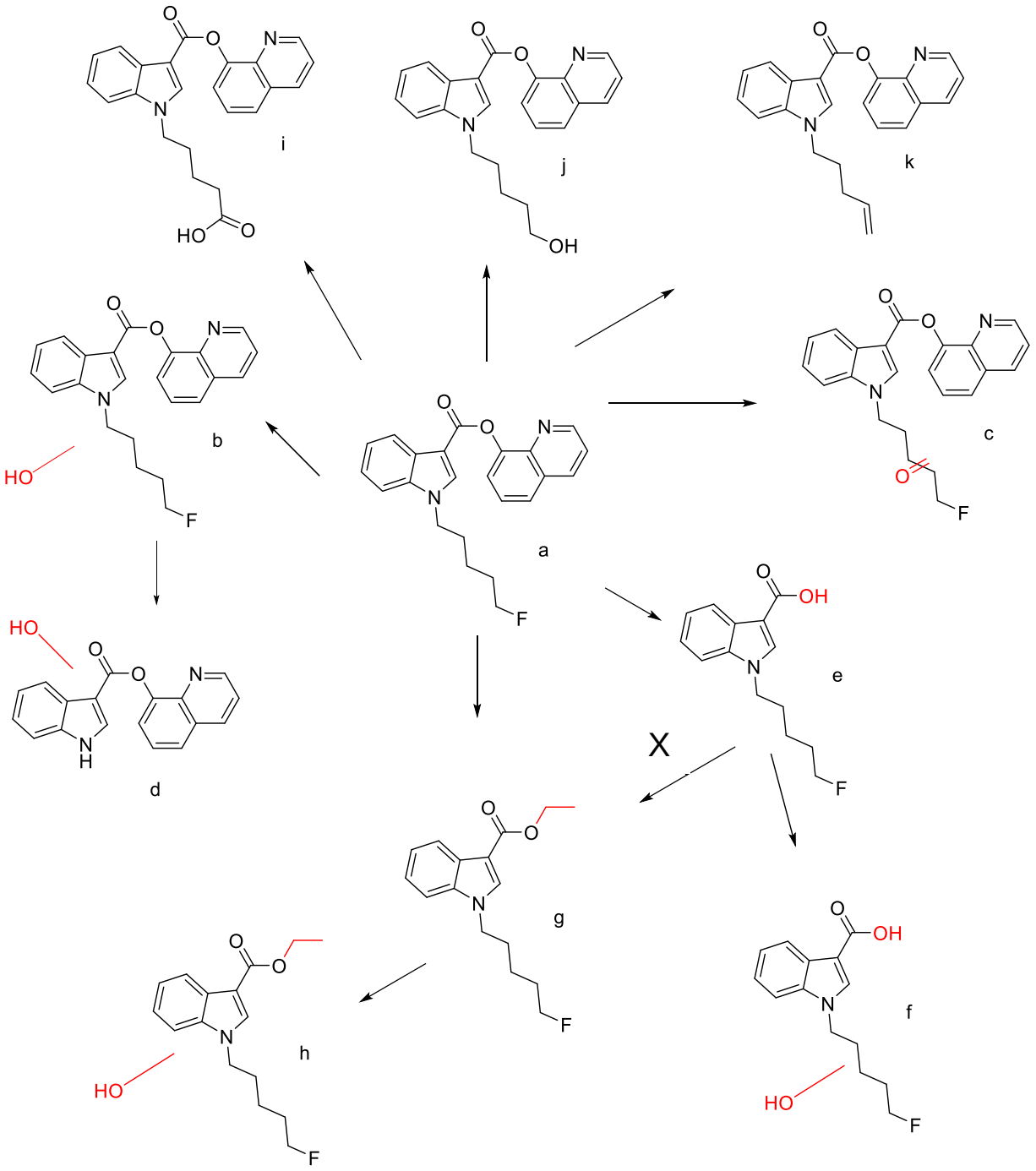


S4: Proposed metabolic pathway of compounds in the presence of ethanol

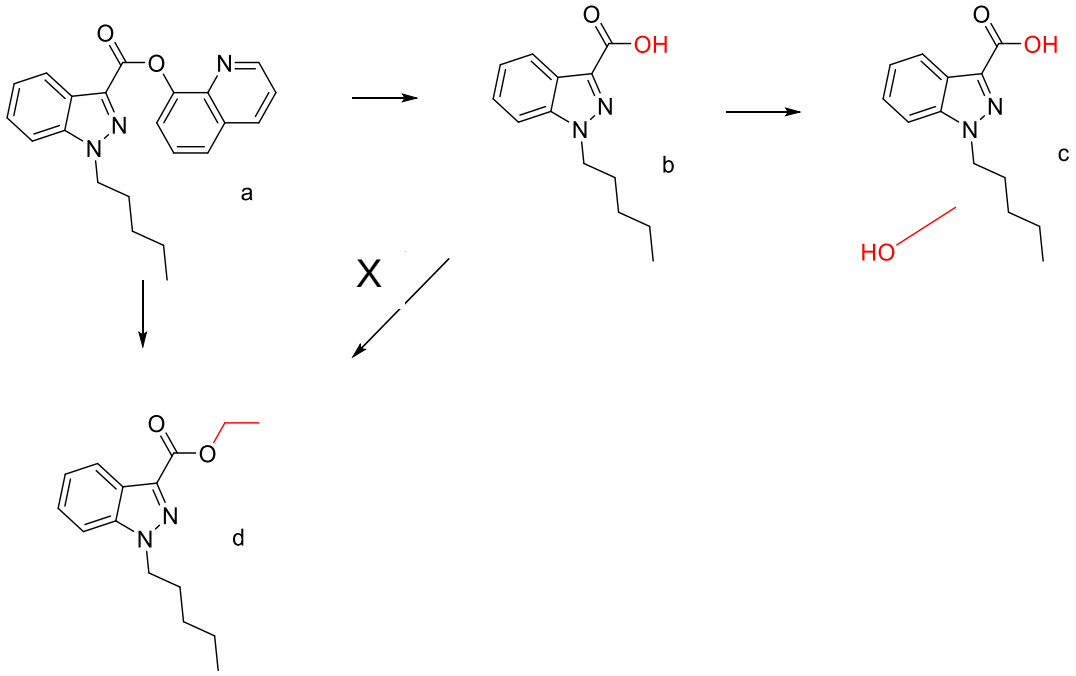
S4.1 Proposed metabolic pathway of PB-22 incubated with HLM and ethanol



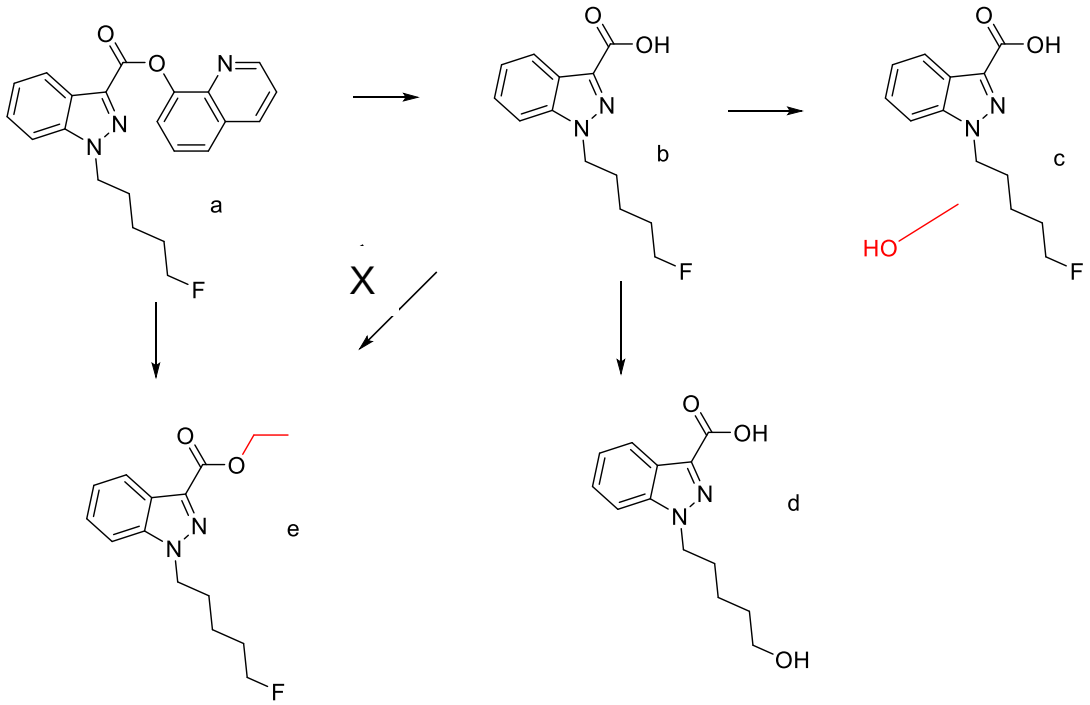
S4.2 Proposed metabolic pathway of 5F-PB-22 incubated with HLM and ethanol



S4.3 Proposed metabolic pathway of NPB-22 incubated with HLM and ethanol



S4.4 Proposed metabolic pathway of 5F-NPB-22 incubated with HLM and ethanol



S5: Biological evaluation of PB-22, NPB-22, 5F-PB-22, 5F-NPB-22, their ethyl esters and their ester hydrolysis products

Table S5.1: EC_{50} and E_{max} values are presented as a measure of potency and efficacy, respectively. Data are given as EC_{50}/E_{max} values (95 % CI profile likelihood).

	CB1 EC_{50} (nM)	**CB1 E_{max} (%)**	**CB2 EC_{50} (nM)**	**CB2 E_{max} (%)**
JWH-018	31.2 (26.0-37.5)	99.8 (96.6-103)	6.38 (4.82-8.48)	99.1 (94.9-104)
PB-22	1.11 (0.78-1.57)	390 (369-414)	1.15 (0.94-1.43)	127 (122-131)
NPB-22	27.0 (19.4-37.8)	326 (309-344)	7.20 (4.53-11.7)	98.5 (90.7-109)
5F-PB-22	1.27 (1.01-1.63)	371 (357-385)	1.38 (1.14-1.70)	111 (107-115)
5F-NPB-22	8.30 (7.42-9.28)	342 (333-350)	3.46 (2.91-4.10)	76.6 (74.4-78.8)

Table S5.2: The maximal effect obtained at CB1. The maximal effect of all compounds was determined by either the E_{max} (for the parent compounds) or the maximal effect that was achieved for the compound (all normalized to the E_{max} of the reference compound JWH-018). Data are given as E_{max} values/maximal receptor activation (95% CI profile likelihood).

	CB1 E_{max} (%) parent compound	**CB1 maximal activation EtOH adduct (1µM)**	**CB1 maximal activation Ester hydrolysis product (10 µM)**
JWH-018	99.8 (96.6-103)	-	-
PB-22	390 (368.6-413.5)	3.24 (2.39-4.08)	0.63 (0.39-0.86)
NPB-22	326 (308.6-344.1)	0.23 (-0.13-0.59)	0.40 (0.27-0.54)
5F-PB-22	371 (356.6-384.9)	4.19 (3.91-4.47)	ND
5F-NPB-22	342 (3.333-350.3)	0.24 (-0.09-0.56)	0.38 (0.19-0.56)

*ND: not determined

Table S5.3: The maximal effect obtained at CB2. The maximal effect of all compounds was determined by either the E_{max} (for the parent compounds) or the maximal effect that was achieved for the compound (all normalized to the E_{max} of the reference compound JWH-018). Data are given as E_{max} values/maximal receptor activation (95% CI profile likelihood).

	CB2 E_{max} (%) parent compound	**CB2 maximal activation EtOH adduct (1 µM)**	**CB2 maximal activation Ester hydrolysis product (10 µM)**
JWH-018	99.1 (94.9-103.6)	-	-
PB-22	127 (122.1-131.4)	35.5 (30.5-40.5)	6.01 (2.92-9.10)
NPB-22	98.5 (90.7-108.9)	11.7 (9.41-14.0)	1.28 (-0.64-3.21)
5F-PB-22	111 (106.9-114.8)	31.7 (22.7-40.8)	ND
5F-NPB-22	76.6 (74.4-78.8)	11.0 (9.92-12.1)	0.58 (-1.84-2.99)

*ND: not determined

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4. Longworth, M.; Banister, S. D.; Mack, J. B. C.; Glass, M.; Connor, M.; Kassiou, M., The 2-alkyl-2H-indazole regioisomers of synthetic cannabinoids AB-CHMINACA, AB-FUBINACA, AB-PINACA, and 5F-AB-PINACA are possible manufacturing impurities with cannabimimetic activities. *Forensic Toxicol.* **2016,** *34*, 286-303.