Phosphotyrosine Prodrugs: Design, Synthesis and Anti-STAT3 Activity of ISS-610 Aryloxy Triester Phosphoramidate Prodrugs[‡]

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Unmasked phohate groups of phosphotyrosine-containing molecules carry two negative charges at physiological pH, which compromise their (passive) cellular uptake. Also, these phosphate groups are often cleaved off by phosphatases. Together, these ultimately limit the pharmacological efficacy of the phosphotyrosine-containing compounds. To address these drawbacks, we herein present the application of the aryloxy triester phosphoramidate prodrug technology, a monophosphate prodrug technology, to the phosphotyrosine-containing compound ISS-610-Met, an analogue of the anticancer STAT3 dimerization inhibitor ISS-610. Our data shows that the generated ISS-610-Met prodrugs exhibited enhanced pharmacological activity and inhibition of STAT3 downstream signaling compared to the parent compound ISS-610-Met and the known STAT3 dimerization inhibitor ISS-610. These encouraging results provide a compelling proof of concept for the potential of the aryloxy triester phosphoramidate prodrug technology in the discovery of novel therapeutics that contain phosphotyrosine and its phospho mimics.

1. Introduction

Phosphotyrosine mediates the formation of protein complexes by binding to various phosphotyrosine-binding domains such as Src homology 2 (SH2) domains,[1] which are present in many proteins that include kinases, phosphatases and transcription factors. Given the vital roles SH2 domains play in mediating many cell signaling events that are implicated in the pathogenesis of human diseases,[2-4] the targeting of these phosphotyrosine-binding domains by small molecules has been noted for a long time as a promising approach in drug discovery.[5] However, the development of these molecules has largely been hindered by the requirement of a phosphotyrosine motif to mimic the endogenous moiety and achieve binding to the target proteins with high affinity. This is because the incorporation of phosphate groups makes these phosphotyrosine-containing molecules unstable *in vivo* due to dephosphorylation by alkyl phosphatases. Additionally, the charged phosphotyrosine species at physiological pH limit their (passive) cellular entry. To address these shortages, phosphate bioisosteres, e.g. carboxylic acids, have been used and this led to a series of compounds with interesting biological activities.[5] However, none of these compounds have progressed to late clinical development.

In order to improve the drug-like properties of phosphotyrosine-containing molecules and their phospho-mimics, we hypothesized that this could be achieved by applying monophosphate prodrug technologies to these compounds. Although there are many prodrug strategies reported to be effective in delivering monophosphorylated molecules into cells, we decided to use the aryloxy triester phosphoramidate prodrug technology[6] as this has already delivered two FDA-approved drugs and over 10 clinical candidates.[7] In this prodrug technology, the monophosphate group is masked by an aryl motif and an amino acid ester (**Fig. 1**). The hydrolysis of these masking groups is now well understood to proceed in vivo via the involvement of two distinct enzymes; carboxypeptidase Y[8] and a phosphoramidase-type enzyme (Hint-1)[9] (**Fig. 1**) [see Mehellou Y *et al*[6] for a detailed account on the aryloxy triester prodrug technology].
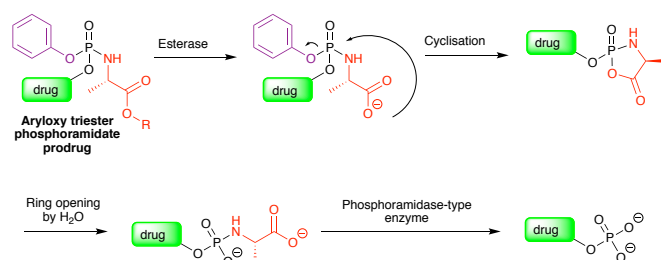

Figure 1. Metabolism of the aryloxy phosphoramidate prodrug technology to release the monophosphate species.

To determine the suitability of the aryloxy triester phosphoramidate prodrug technology, we chose the STAT3 dimerization inhibitor ISS-610, a peptidomimetic that contains a phosphotyrosine motif (**Fig. 2**). ISS-610 was first reported in 2004 as a binder of a transcription factor known as the signal transducer and activator of transcription 3 (STAT3).[10] This molecule contains a phosphotyrosine moiety by design, which allows it to bind the SH2 domain of STAT3 resulting in the inhibition of its dimerization and downstream signaling. The interest in the discovery of small molecules that inhibit STAT3 is driven by the fact that STAT3 is constitutively active in numerous cancers and contributes to tumor cell proliferation and survival.[11] At the molecular level, the activation of STAT3 involves phosphorylation by upstream kinases, which leads to its homo- and heterodimers with

other STAT isoforms. This dimerization is critical for its downstream signaling and thus the inhibition of this dimerization process emerged as a favorable approach in the discovery of anticancer STAT3 inhibitors.
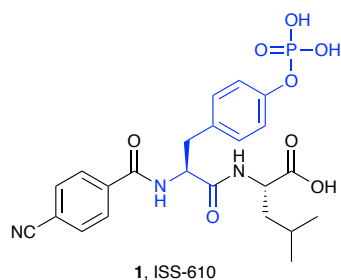

1, ISS-610

Figure 2. Chemical structure of the phosphotyrosine containing STAT3 dimerization inhibitor ISS-610.

ISS-610 was found to be able to inhibit the binding of STAT3 to the target DNA with moderate efficacy, IC_{50} = 42 μM.[10] Although cellular data also supported the ability of ISS-610 to inhibit STAT3 dimerization and subsequent downstream signaling, the concentrations required to achieve these in cells were relatively high (~ 1 mM).[10] We suspected that this was due to poor cellular uptake of ISS-610 that results from its relatively large size, charged nature at physiological pH and dephosphorylation of the phosphotyrosine moiety. These limitations made ISS-610 an ideal candidate for the application of the powerful monophosphate prodrug technology, the aryloxy triester phosphoramidate technology.

2. Results and Discussion

2.1. Synthesis of ISS-610 and its prodrugs

In the design of ISS-610 phosphoramidate prodrugs, the carboxylic acid of ISS-610 had to be protected to avoid a nucleophilic attack from the free carboxylate group onto the phosphorous atom leading to the breakdown of the prodrug motif akin to its in vivo metabolism. Thus, ISS-610 prodrugs presented in this work carry a methyl group that blocks the *C*-terminal carboxylic group of the *L*-leucine amino acid, a compound we termed ISS-610-Met. As a control, we also synthesised the ISS-610-Met monophosphate (**7**) (**Figure 3**). Notably, modifications of ISS-610 in its *C*-terminal region have been extensively explored and led to the discovery of potent STAT3 dimerization inhibitors.[12]

The synthesis of the ISS-610-Met started by the coupling of the Boc-tyrosine (**2**) and *L*-leucine methyl ester via the use of the peptide coupling reagent benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (PyBOP) in the presence of triethylamine (TEA) to attain dipeptide **3** in a high yield (94%). Removal of the Boc-protecting group with trifluoroacetic acid (TFA) afforded fragment **4** in almost quantitative yield (99%) and this was subsequently coupled to 4-cyanobenzoyl chloride, which had been prepared as reported[13]. Subsequently, the phosphorodiamidate of compound **5** was synthesised by reacting intermediate **5** with *N,N,N',N'*-tetramethylphosphorodiamidic chloride in the presence of DMAP and DBU to generate the desired product, **6**, in a moderate yield, 50%.[12] Finally, the acidification diamidate **6** with TFA yielded acid ISS-610-Met (**7**) in high yield, 76%.[12]

Next, we synthesised ISS-610-Met aryloxy triester phosphoramidate prodrugs (**Figure 4**), the phosphorochlorides were initially synthesised as previously reported.[14, 15] Briefly, 1-napthol (**8**) in dry diethyl ether at -78 °C was treated with phosphorous oxychloride

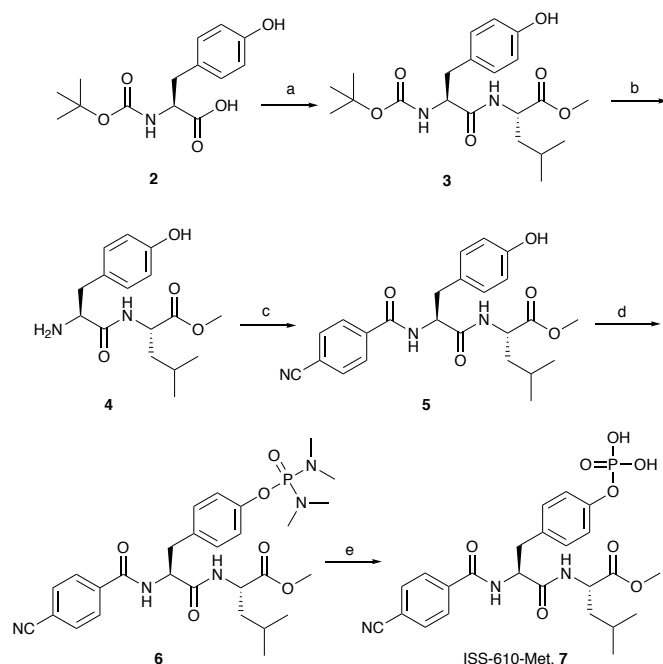**Figure 3. Synthesis of ISS-610-Met (7).** Reagents and conditions: *a)* PyBop, *L*-leucine methyl ester hydrochloride, NEt_3, THF, 94%; b) TFA, DCM, 99%; c) 4-cyanobenzoyl chloride, NEt_3, THF, 42%; d) *N,N,N',N'*-tetramethylphosphorodiamidic chloride, DMAP, DBU, THF, 50%; e) TFA, H_2O, 76%.

($POCl_3$) and TEA to afford phosphorodichloridate **9**, which was used in the next step without purification. Subsequently, this was dissolved in anhydrous DCM and reacted with the desired *L*-amino acid esters in the presence of triethylamine to give the desired phosphorochloridates in excellent yields (70-97%).

The coupling of the phosphorochloridates, **10**-**12**, to the ISS-610-Met core structure, **5**, was pursued in dry THF using triethylamine as a base. This gave the desired ISS-610-Met phosphoramidate prodrugs, **13**-**15**, in good yields (72-93%). Notably, the final prodrugs, **13**-**15**, were generated as a mixture of two diastereoisomers due to the chiral phosphorous center and as indicated by the phosphorous ^{31}P-NMR of these compounds (see Supplementary Information). This is typical for the synthesis of aryloxy triester phosphoramidate prodrugs and many of these prodrugs are synthesised and studied as a mixture of diastereoisomers[6] with some of them even undergoing (late) clinical trials in this form, e.g. NUC-1031[15].
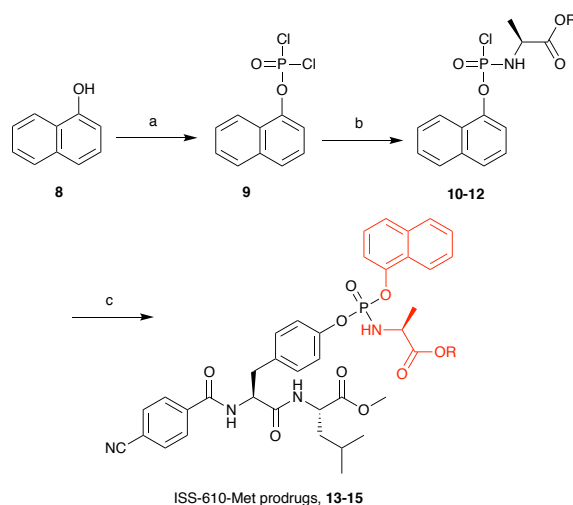

ISS-610-Met prodrugs, **13-15**

Figure 4. Synthesis of ISS-610-Met aryloxy triester phosphoramidate prodrugs. Reagents and conditions: a) $POCl_3$, TEA, diethyl ether, -78°C, 98%; b) for compound **10** (R = methyl): *L*-alanine methyl ester hydrochloride, TEA, DCM, -78 °C, 70%, for compound **11** (R = isopropyl): *L*-alanine isopropyl ester hydrochloride, TEA, DCM, -78 °C, 72%, for compound **12** (R = benzyl): *L*-alanine benzyl ester hydrochloride, TEA, DCM, -78 °C, 97%; c) for compound **13** (R = methyl): phosphorochloridate **10**, TEA, DCM, 72%, for compound **14** (R = isopropyl): phosphorochloridate **11**, TEA, DCM, 91%, for compound **15** (R = benzyl): phosphorochloridate **12**, TEA, DCM, 93%;

In the design of the aryloxy triester phosphoramidate prodrugs, the most common aryl motif used is the phenyl followed by naphthyl. In this work, we chose the naphthyl as the aryl motif of the prodrug moiety as it is a better leaving group than phenol and hence in the metabolism of the prodrugs the -P-O- bond would favor cleavage to release the 1-naphthol group (Option 1, **Figure 5**) instead of cleaving the bond to release the phenol of the phosphotyrosine (Option 2, **Figure 5**). Indeed, following entry to cells, the ester group of the aryloxy triester phosphoramidate prodrugs is cleaved off by esterases, e.g. carboxypeptidase Y, to yield an unmasked carboxylate group (**16**). This then performs a nucleophilic attack on the phosphorous center. This leads to the cleavage of one of the -O-P-phosphorous bonds as labelled options 1 and 2 in **Figure 5**. Thus, in this case, having a naphthyl group as a leaving group would favor option 1 and hence lead to the formation of the highly unstable anhydrous intermediate **17**. This subsequently gets opened up by a nucleophilic attack from a water molecule on the phosphorous

center generating phosphoramidate **18**. Finally, a phosphoramidase-type enzyme. E.g. Hint-1, cleaves the P-N bond to release the unmasked monophosphate **19**, ISS-610-Met in this case.
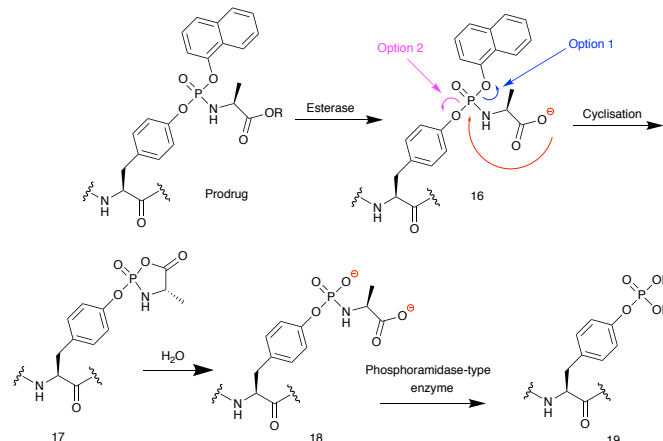

Figure 5. Postulated mechanism of aryloxy triester phosphoramidate prodrugs.[6]

2.2. Serum stability and metabolism of ISS-610 prodrugs

As compounds **13**-**15** are prodrugs, we initially studied their stability in human serum. For this prodrug **14** was incubated with human serum at 37 °C for 12 h and the mixture was monitored by ^{31}P-NMR
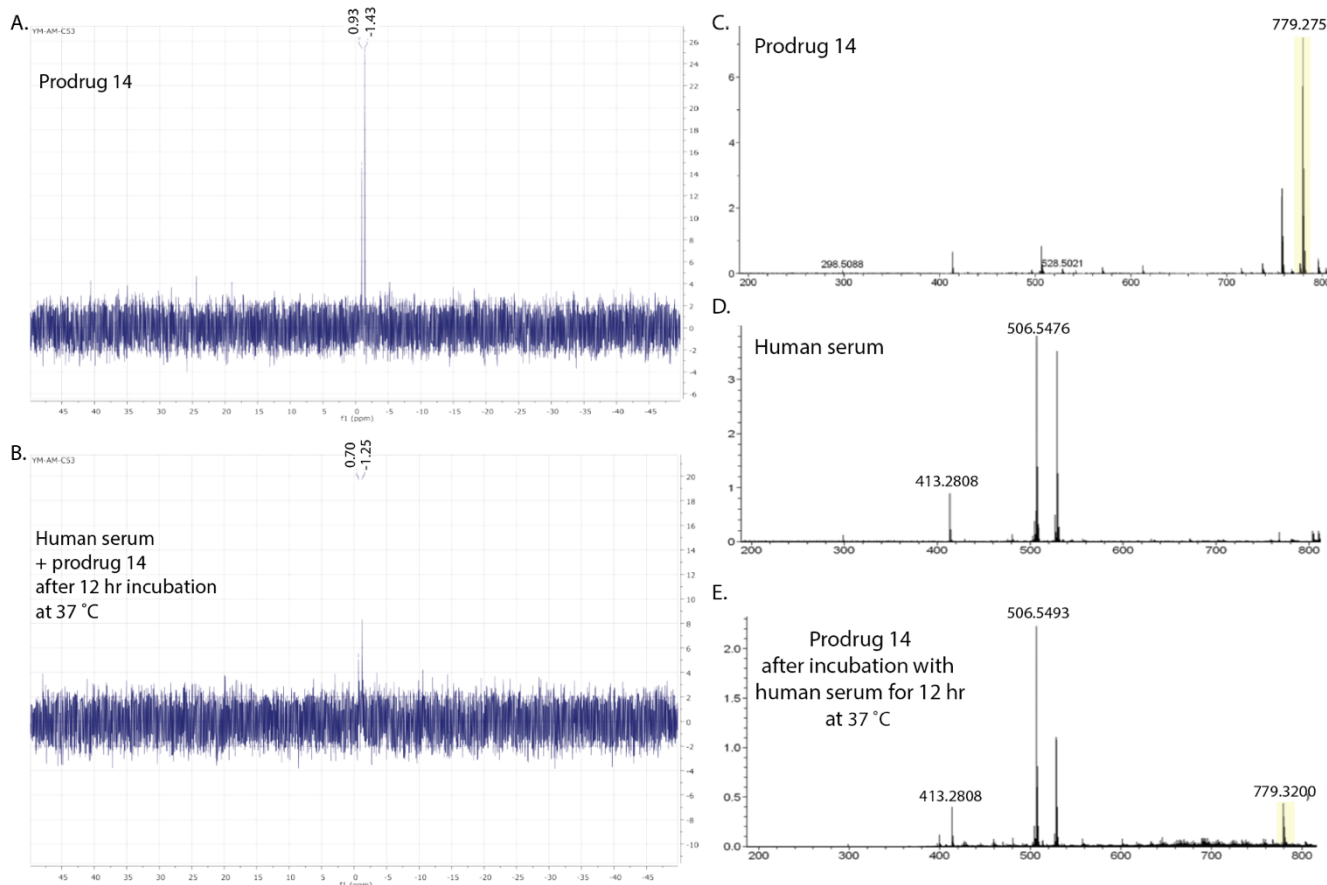
Figure 6. Human serum stability of Prodrug 14. A: ^{31}P-NMR scan of prodrug **14** in 0.15 mL DMSO and 0.15 mL D_2O. δ_P = -1.43 and 0.93. **B**: Final ^{31}P-NMR scan of the sample from **A** after being incubated with 0.3 mL of human serum at 37 °C for 12 h. **C**: LCMS trace of prodrug **14** alone. The peak corresponding to the mass of the parent compound, 779.2750 g/mol (+Na), is highlighted in light yellow. **D**: LCMC trace the human serum alone. **E**: LCMS trace of the organic fraction of the sample of prodrug **14** after being incubated with 0.3 mL of human serum at 37 °C for 12 h. The peak corresponding to the mass of the parent compound, 779.2750 g/mol (+Na), is highlighted in light yellow.

Figure 7. Inhibition of the STAT3-dependent expression of the anti-apoptotic proteins Survivin and Bcl-xl by ISS-610 prodrugs. Cultured MDA-MB-468 cells were treated with DMSO (control), ISS-610, prodrugs 13-15 at the indicated concentrations for 24 h. The cell lysates subsequently underwent Western blotting for total STAT3, total Survivin and total Bcl-xl. Total β-actin was included as a loading control.

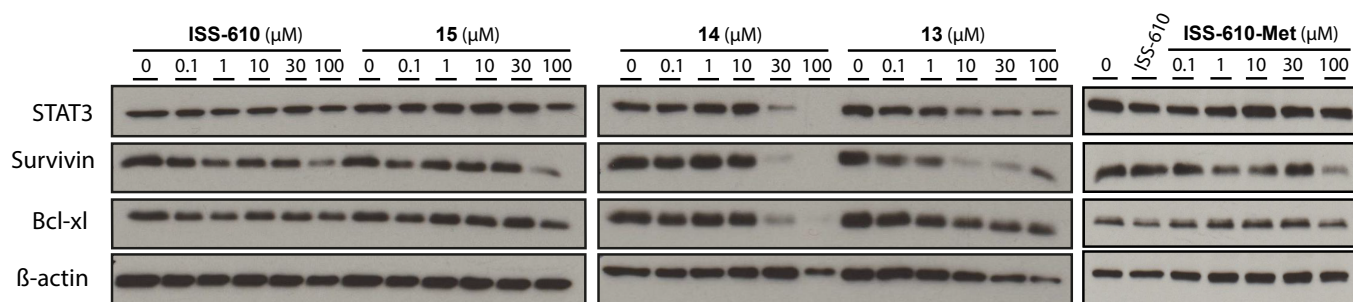as we reported previously.[14] At t = 0 h, two singlets appeared on the ^{31}P-NMR, δ_P = -1.43 and 0.93, which correspond to the two diastereoisomers of prodrug 13 (**Figure 6**). Upon the addition of the human serum and monitoring the reaction for 12 h, no new phosphorous peaks were detected indicating the stability of this prodrug in human serum. Although the intensity of the prodrug peaks at t = 12 h appeared lower than those at t = 0 h, this was due to some of the prodrug crashing out of solution following the addition of the serum (**Figure 6**). This is due to the relatively high lipophilicity of prodrug 13 (cLog P = 6.06). This is a common observation is studying these prodrugs when they have a relatively high lipophilicity. To further confirm the stability of prodrug 13, we run a mass spectrometry of the parent compound by itself and then the sample following 12 h incubation in human serum. The results showed that no new mass spectrometry peaks appeared following incubation with human serum for 12 h. This is a further confirmation of the stability of this prodrug in human serum for the 12 h studied. Notably, this stability profile is in line with the human serum stability profiles of the aryloxy triester phosphoramidate prodrugs of nucleoside monophosphates.[14, 15]

2.3. Effect of ISS-610 and ISS-610-Met prodrugs on cancer cells

The activation of the JAK/STAT3 signalling cascade leads to STAT3 phosphorylation, dimerization and translocation to the nucleus where it mediates the transcription of anti-apoptotic genes such as Survivin and Bcl-xl.[16] Hence, in determining the potency of our prodrugs' inhibition of STAT3 dimerization, we probed for the expression levels of Survivin and Bcl-xl. For this, we chose the breast cancer cell line MDA-MB-468, which has the JAK/STAT3 signalling cascade constitutively active and is widely used in studying the biological activity of STAT3 inhibitors.[17-19] Thus, the cells were treated with the parent compound ISS-610 (**1**) or its *C*-methylated derivative ISS-610-Met (**7**) as positive controls, or prodrugs 13-15 at the indicated concentrations for 24 hours. Following lysis, the cell lysates underwent Western blotting for total Survivin and Bcl-xl as a readout of the compound's ability to inhibit STAT3 dimerization and downstream signalling. As shown in **Figure 7**, the parent compounds, ISS-610 and ISS-610-Met, did not have any significant downregulation of the expression of the anti-apoptotic proteins Survivin and Bcl-xl, which is in line with the poor cell activity of the ISS-610 peptidomimetic.[10] However, prodrugs 13-15 showed more profound downregulation of the expression of Survivin and Bcl-xl. This was most notable with compounds 13 and 14, which exhibited significant reduction of Survivin even at 30 μM. In terms of the structure-activity relationship of aryloxy triester phosphoramidate prodrugs, the ones with benzyl ester tend to exhibit more potent biological activity than the ones with methyl and isopropyl esters.[6] However, in this work, the prodrugs with methyl and isopropyl

esters, 13 and 14 respectively, exerted more potent activity than their prodrug counterpart with the benzyl ester, compound 15. This is most likely due to the relatively high lipophilicity of compound 15 as compared to compounds 13 and 14, which could limit their solubility in the cell media. Indeed, in treating the cells with prodrugs 13-15, only prodrug 15 upon addition to the cells resulted in a cloudy media, which rapidly cleared up after gentle shaking. Nevertheless, the observed downregulation of Survivin and Bcl-xl with prodrugs 13 and 14 relative to the lack of any effect on these proteins by the parent compounds ISS-610 and ISS-610-Met was notable and indicates the potential of this prodrug approach in increasing the potency of phosphotyrosine-containing STAT3 dimerization inhibitors.

3. Conclusion

STAT3 dimerization is an essential process for its downstream signalling and cancer cell survival. The inhibition of this process using phosphotyrosine-containing small molecules that bind the STAT3 SH2 domain is an attractive approach in treating cancer. As small molecules that contain phosphotyrosine and its phospho mimics often have poor cellular uptake, we herein described – as a proof of concept- the application of the aryloxy triester phosphoramidate approach to ISS-610-Met, an analogue of phosphotyrosine-containing STAT3 dimerization inhibitor ISS-610. Our data showed that these prodrugs are stable in human serum at 37 °C for 12 h. In cells, these prodrugs were relatively more potent in inhibiting STAT3 downstream signalling than their parent compounds ISS-610 and ISS-610-Met. Collectively, this work highlights the novel applicability of the aryloxy triester phosphoramidate prodrug approach in discovering new therapeutics that contain phosphotyrosine- or its phospho mimics, an endeavour that has proved to be a stern challenge so far.

4. Experimental

General Information. Dichloromethane, diethyl ether, methanol, and toluene were dried in-house using a Pure Solv-MD solvent purification system. All the other solvents were used as received from commercial suppliers. All of the other reagents used in the synthesis were purchased from Sigma-Aldrich except *L*-alanine isopropyl ester, which was synthesised in house. ISS-610 was purchased from Alta Biosciences (Birmingham, UK) and it had a 99.2% purity. All reactions were carried out under an argon atmosphere. Reactions were monitored with analytical TLC on silica gel 60-F254 precoated aluminum plates and visualized under UV (254 nm) and/or with 31P NMR spectra. Column chromatography was performed on silica gel (35-70 μM). NMR data were recorded on a Bruker AV300, AVIII300, AV400, AVIII400, or DRX500 spectrometer in

the deuterated solvents indicated, and the spectra were calibrated on residual solvent peaks. Chemical shifts (δ) are quoted in ppm, and J values are quoted in Hz. In reporting spectral data, the following abbreviations were used: s (singlet), d (doublet), t (triplet), q (quartet), dd (doublet of doublets), and m (multiplet). HPLC was carried out on a DIONEX summit P580 quaternary low-pressure gradient pump with a built-in vacuum degasser using a Summit UVD 170s UV/vis multichannel detector. Solvents were used as HPLC grade. Chromeleon software was used to visualize and process the obtained chromatograms. Analytical separations used a flow rate of 1 mL/min, semipreparative used a flow rate of 3 mL/min, and preparative used a flow rate of 20 mL/min. All tested compounds had a purity of ≥95% unless otherwise stated (see Supporting Information).

(S)-methyl 2-((S)-2-((tert-butoxycarbonyl)amino)-3-(4-hydroxyphenyl)propanamido)-4-methylpentanoate (3). To a stirring, colorless solution of Boc-L-tyrosine 2 (125 mg, 0.444 mmol), in dry THF (5 mL), was added PyBOP (277 mg, 0.532 mmol), L-leucine methyl ester hydrochloride (162 mg, 0.889 mmol) and triethylamine (0.19 mL, 1.332 mmol). The white suspended solution was stirred at room temperature for eight hours under an argon atmosphere. After reaction completion, the orange solution was concentrated under reduced pressure and diluted with water (50 mL), where the product was extracted with ethyl acetate (3x50 mL). The combined organic layers were concentrated to 50 mL and washed with water (1x50 mL) and brine (1x50 mL), dried with $MgSO_4$, filtered and concentrated under reduced pressure to afford a colorless oil. Flash column chromatography (2:1 ethyl acetate: hexane) was utilized to purify the product and yield dipeptide 3 as a white crystalline solid (170 mg, 94%). ^{1}H NMR (500 MHz, $CDCl_3$) δ = 7.03 (d, J = 8.2 Hz, 2H), 6.73 (d, J = 8.5 Hz, 2H), 6.48 (s, 1H), 6.34 (d, J = 8.2 Hz, 1H), 5.11 (s, 1H), 4.60 – 4.50 (m, 1H), 4.28 (s, 1H), 3.69 (s, 3H), 2.97 (d, J = 6.8 Hz, 2H), 1.62 – 1.52 (m, 2H), 1.50 – 1.45 (m, 1H), 1.43 (s, 9H), 0.90 (t, J = 6.6 Hz, 6H). ^{13}C NMR (101 MHz, $CDCl_3$) δ = 172.9, 171.7, 155.4, 130.4, 127.5, 115.6, 80.5, 55.9, 52.3, 50.8, 41.4, 37.4, 28.2, 24.6, 27.7, 21.8. MS (ES) 431.2 [M+Na]+, 331.2 [M-Boc+Na]+.

(S)-methyl 2-((S)-2-amino-3-(4-hydroxyphenyl)propanamido)-4-methylpentanoate (4). To a stirring solution of compound 3 (350 mg, 0.857 mmol) in DCM (8 mL), TFA (2.5 mL) was carefully added. The orange solution was left to stir at room temperature for two hours. Upon reaction completion, the orange solution was concentrated under reduced pressure, diluted with water (50 mL) and neutralized to pH 8 with 10% NaOH. The product was extracted from the orange solution with ethyl acetate (4x50 mL). The combined organic layers were dried with $MgSO_4$, filtered and concentrated under reduced pressure to yield peptide 4 as a cream/white solid (260 mg, 99%). ^{1}H NMR (500 MHz, MeOD) δ = 7.14 (d, J = 8.4 Hz, 2H), 6.79 (d, J = 8.4 Hz, 2H), 4.52 (t, J = 7.3 Hz, 1H), 4.13 – 3.99 (m, 1H), 3.73 (s, 3H), 3.24 – 2.87 (m, 2H), 1.75 – 1.58 (m, 3H), 0.97 (dd, J = 15.8, 6.1 Hz, 6H). ^{13}C NMR (126 MHz, MeOD) δ = 172.5, 168.7, 156.8, 130.3, 124.6, 115.4, 54.4, 50.9, 40.2, 36.5, 24.5, 21.8, 20.4. MS (ES) m/z 317.1 [M+Na]+, 295.1 [M+H]+.

(S)-2-((S)-2-(4-cyanobenzamido)-3-(4-hydroxyphenyl)propanamido)-4-methylpentanoic acid (5). Compound 4 (118 mg, 0.382 mmol) was dissolved in THF (4 mL) and triethylamine (0.11 mL, 0.764 mmol) and stirred under an argon atmosphere at room temperature for 30 minutes. To the colorless solution, 4-cyanobenzyl acid chloride[13] (70 mg, 0.421 mmol) in THF (2 mL) was added dropwise and left to stir overnight at room temperature. The cream solution, with a suspension of white precipitate, was concentrated under reduced pressure and then diluted in water (20 mL), where the product was extracted with ethyl

acetate (3x20mL). The combined orange organic layers were washed with water (20 mL) and brine (20 mL), dried with $MgSO_4$, filtered and concentrated under reduced pressure to afford an orange oil. Column chromatography (2:1 ethyl acetate: hexane) was used to yield dipeptide 5 as white crystals (70 mg, 42%). ^{1}H NMR (300 MHz, $CDCl_3$) δ = 7.84 (d, J = 8.5 Hz, 2H), 7.73 (d, J = 8.5 Hz, 2H), 7.13 (d, J = 8.5 Hz, 2H), 7.04 (d, J = 7.5 Hz, 1H), 6.75 (d, J = 8.5 Hz, 2H), 6.07 (d, J = 7.9 Hz, 1H), 5.49 (s, 1H), 4.87 – 4.76 (m, 1H), 4.59 – 4.49 (m, 1H), 3.75 (s, 3H), 3.12 (ddd, J = 21.6, 13.9, 6.7 Hz, 2H), 1.58 – 1.39 (m, 3H), 0.89 (d, J = 5.6 Hz, 6H). ^{13}C NMR (101 MHz, $CDCl_3$) δ = 172.8, 171.2, 165.5, 155.3, 137.4, 132.4, 130.5, 127.9, 122.3, 117.9, 115.6, 55.0, 52.5, 51.2, 41.2, 37.8, 24.8, 22.6, 21.9. MS (ES) m/z 460.2 [M+Na]+.

Methyl ((S)-3-(4-((bis(dimethylamino)phosphoryl)oxy)phenyl)-2-(4-cyanobenzamido)propanoyl)-L-leucinate (6) To a stirring solution of compound 5 (100 mg, 0.229 mmol), under a nitrogen atmosphere in anhydrous THF (20 mL), was added N,N,N',N'-tetramethylphosphorodiamidic chloride (0.07 mL, 0.457 mmol), DMAP (56 mg, 0.457 mmol) and DBU (0.05 mL, 0.344 mmol). The orange solution was stirred at room temperature overnight. Upon reaction completion the mixture was quenched with methanol (10 mL) and stirred for 10 minutes. The solvent was then removed under reduced pressure and the resultant orange oil was subjected to silica flash column chromatography (3:97 methanol: DCM) to isolate diamidate 6 as a white solid (65 mg, 50%). ^{1}H NMR (500 MHz, $CDCl_3$) δ = 7.79 (d, J = 8.4 Hz, 2H), 7.65 (d, J = 7.6 Hz, 1H), 7.62 (d, J = 8.4 Hz, 2H), 7.18 (d, J = 8.5 Hz, 2H), 7.06 (d, J = 7.8 Hz, 2H), 7.02 (d, J = 7.9 Hz, 1H), 4.89 (q, J = 6.9 Hz, 1H), 4.55 (td, J = 8.5, 5.3 Hz, 1H), 3.73 (s, 3H), 3.11 (ddd, J = 35.6, 14.1, 6.7 Hz, 2H), 2.69 (dd, J = 15.7, 10.1 Hz, 12H), 1.68 – 1.56 (m, 2H), 1.53 (ddd, J = 12.0, 9.0, 4.5 Hz, 1H), 0.87 (dd, J = 6.1, 4.7 Hz, 6H). ^{13}C NMR (126 MHz, $CDCl_3$) δ = 172.8, 170.8, 165.5, 150.3, 137.7, 132.5, 132.2, 130.7, 127.9, 120.2, 118.0, 115.1, 54.8, 52.3, 51.1, 41.1, 37.1, 36.7, 24.8, 22.7, 21.8. ^{31}P NMR (202 MHz, $CDCl_3$) δ = 15.79. **MS** (ES) m/z 594.3 [M+Na]+.

Methyl ((S)-2-(4-cyanobenzamido)-3-(4-(phosphonooxy)phenyl)propanoyl)-L-leucinate (7) A stirring solution of diamidate 6 (60 mg, 0.102 mmol) in 9:1 TFA (9 mL): H_2O (1 mL) was stirred overnight at ambient temperature. Upon reaction completion, the solvent was removed to afford a cream solid. The solid was further dried under vacuum until constant weight, yielding acid 7 as a white solid (40 mg, 76%). The compound was used without purification. ^{1}H NMR (500 MHz, MeOD) δ = 7.74 (d, J = 8.1 Hz, 2H), 7.70 (d, J = 8.1 Hz, 2H), 7.15 (d, J = 7.2 Hz, 2H), 7.05 (d, J = 7.6 Hz, 2H), 4.39 (dd, J = 9.4, 5.6 Hz, 1H), 3.86 – 3.75 (m, 1H), 3.60 (s, 3H), 3.01 (ddd, J = 23.9, 14.3, 7.5 Hz, 2H), 1.62 (dd, J = 12.5, 6.6 Hz, 1H), 1.58 – 1.50 (m, 2H), 0.83 (dd, J = 22.1, 6.5 Hz, 6H). ^{13}C NMR (126 MHz, MeOD) δ = 173.0, 172.4, 167.1, 138.2, 132.1, 132.0, 131.9, 129.7, 128.0, 120.0, 117.6, 114.7, 55.2, 51.4, 50.9, 40.1, 36.5, 24.5, 21.9, 20.4. ^{31}P NMR (202 MHz, MeOD) δ = -4.34. MS (ES) m/z 516.3 [M-H]-. HRMS $C_{24}H_{27}N_3O_8NaP$ calcd. 516.1541 [M-H]-, found 516.1541.

Naphthalen-1-yl phosphorodichloridate (9). In a flame dried flask, $POCl_3$ (0.56 mL, 6.588 mmol) was added to a solution of 1-naphthol (864 mg, 6 mmol) in dry diethyl ether (6 mL). The colorless solution was stirred at room temperature for 30 minutes under an atmosphere of argon. The solution was then cooled to -78 °C, where triethylamine (0.84 mL, 6.588 mmol) was added dropwise and then allowed to stir for 30 minutes, before being left to stir for one hour at room temperature. The white precipitate was filtered out of the solution and washed with diethyl ether. The yellow filtrate was concentrated under reduced pressure to afford phosphorochloridate 9 as a yellow oil (1.54 g, 98%), which was used without purification.

Methyl (chloro(naphthalen-1-yloxy)phosphoryl)-L-alaninate (10). To a stirring solution of compound 9 (513 mg, 1.974 mmol) and dry

DCM (5 mL), in a flame dried flask, was added L-alanine methyl ester hydrochloride (250 mg, 1.792 mmol) and stirred for 15 minutes at room temperature, under an argon atmosphere. Triethylamine (0.5 mL, 3.585 mmol) was added dropwise to the yellow solution at -78°C to form a suspended white precipitate. The reaction mixture was stirred for 30 minutes before being left to stir and warm to room temperature for 2.5 hours. Upon reaction completion, the grey/white solution was concentrated under reduced pressure and filtered with ether. The yellow filtrate was concentrated under reduced pressure to afford a yellow oil, which was purified with column chromatography (6:4 ethyl acetate: hexane) to yield phosphoramidate **10** as a yellow oil (411 mg, 70%). ^{1}H NMR (300 MHz, CDCl$_3$) δ = 8.10 – 8.02 (m, 1H), 7.88 (d, J = 7.5 Hz, 1H), 7.74 (d, J = 8.2 Hz, 1H), 7.64 – 7.50 (m, 3H), 7.44 (t, J = 7.9 Hz, 1H), 4.45 – 4.24 (m, 2H), 3.79 (d, J = 14.2 Hz, 3H), 1.55 (dd, J =9.2, 5.3 Hz, 3H). ^{31}P NMR (121 MHz, CDCl$_3$) δ = 8.2, 7.9.

Isopropyl (chloro(naphthalen-1-yloxy)phosphoryl)-*L*-alaninate (11)
To a stirring solution of compound **9** (513 mg, 1.97 mmol) and dry DCM (5 mL), in a flame dried flask, was added L-alanine isopropyl ester hydrochloride (300 mg, 1.794 mmol) and stirred for 15 minutes at room temperature, under an argon atmosphere. Triethylamine (0.5 mL, 3.581 mmol) was added dropwise to the yellow solution at -78°C to form a suspended white precipitate. The reaction mixture was stirred for 30 minutes before being left to stir and warm to room temperature for 2.5 hours. Upon reaction completion, the grey/white solution was concentrated under reduced pressure and filtered with ether. The yellow filtrate was concentrated under reduced pressure to afford a yellow oil, which was purified with column chromatography (6:4 ethyl acetate: hexane) to yield compound **11** as a yellow oil (504 mg, 72%). ^{1}H NMR (300 MHz, CDCl$_3$) δ = 8.07 (dd, J = 6.9, 4.7 Hz, 1H), 7.87 (t, J = 3.0 Hz, 1H), 7.74 (d, J = 8.3 Hz, 1H), 7.62 – 7.51 (m, 3H), 7.44 (t, J = 8.0 Hz, 1H), 5.09 (tt, J = 17.1, 6.3 Hz, 1H), 4.47 – 4.16 (m, 2H), 1.54 (t, J = 6.5 Hz, 3H), 1.32 – 1.24 (m, 6H). ^{31}P NMR (121 MHz, CDCl$_3$) δ = 8.3, 7.9.

Benzyl (chloro(naphthalen-1-yloxy)phosphoryl)-*L*-alaninatealaninate (12) To a stirring solution of compound **9** (513 mg, 1.972 mmol) and dry DCM (5 mL), in a flame dried flask, was added L-alanine benzyl ester hydrochloride (386 mg, 1.794 mmol) and stirred for 15 minutes at room temperature, under an argon atmosphere. Triethylamine (0.5 mL, 3.583 mmol) was added dropwise to the yellow solution at -78°C to form a suspended white precipitate. The reaction mixture was stirred for 30 minutes before being left to stir and warm to room temperature for 2.5 hours. Upon reaction completion, the grey/white solution was concentrated under reduced pressure and filtered with ether. The yellow filtrate was concentrated under reduced pressure to afford a yellow oil, which was purified with column chromatography (6:4 ethyl acetate: hexane) to yield compound **12** as a yellow oil (700 mg, 97%). ^{1}H NMR (300 MHz, CDCl$_3$) δ = 8.09 – 7.98 (m, 1H), 7.87 (dd, J = 7.8, 3.5 Hz, 1H), 7.73 (d, J = 8.3 Hz, 1H), 7.61 – 7.52 (m, 3H), 7.44 (d, J = 7.9 Hz, 1H), 7.40 – 7.30 (m, 5H), 5.18 (dd, J = 16.4, 11.9 Hz, 2H), 4.44 – 4.23 (m, 2H), 1.56 (t, J = 6.6 Hz, 3H). ^{31}P NMR (121 MHz, CDCl$_3$) δ = 8.1, 7.8.

Methyl ((2*S*)-2-(4-cyanobenzamido)-3-(4-(((((*S*)-1-methoxy-1-oxopropan-2-yl)amino)(naphthalen-1-yloxy)phosphoryl)oxy)phenyl)propanoyl)-*L*-leucinate (13) In a flame dried flask, a solution of compound **5** (100 mg, 0.229 mmol) in dry DCM (3 mL) was stirred with dry triethylamine (0.07 mL, 0.504 mmol) at room temperature for 15 minutes under an argon atmosphere. Phosphorochloridate **10** (113 mg, 0.344 mmol) was added dropwise in DCM (2 mL). The yellow solution was stirred at room temperature for 5 hours; where it was then concentrated under reduced pressure to form a dense yellow oil. The oil was

diluted in water (20 mL) and the product was extracted with ethyl acetate (3x20 mL), where the yellow combined organic layers were washed with water (20 mL) and brine (20 mL), dried with MgSO$_4$, filtered and concentrated to form a white solid. Column chromatography (2:1 ethyl acetate: hexane) was used to isolate prodrug **13** as white crystals (120 mg, 72%). ^{1}H NMR (400 MHz, CDCl$_3$) δ = 8.00 (dd, J = 12.5, 6.6 Hz, 1H), 7.83 (dd, J = 10.6, 7.8 Hz, 1H), 7.72 (dd, J = 14.6, 7.8 Hz, 2H), 7.65 (dd, J = 8.0, 4.7 Hz, 1H), 7.57 – 7.43 (m, 5H), 7.41 – 7.30 (m, 1H), 7.22 – 7.04 (m, , 4H), 6.94 (d, J = 7.9 Hz, 1H), 4.97 – 4.82 (m, 2H), 4.55 (dd, J = 13.1, 8.1 Hz, 1H), 4.50 – 4.40 (m, 1H), 4.25 – 4.09 (m, 1H), 3.67 (d, J = 6.4 Hz, 3H), 3.57 (d, J = 16.3 Hz, 3H), 3.23 – 3.01 (m, 2H), 1.67 – 1.43 (m, 3H), 1.36 (dd, J = 22.2, 7.0 Hz, 3H), 0.82 (d, J = 5.8 Hz, 6H). ^{13}C NMR (101 MHz, CDCl$_3$) δ = 173.6, 172.9, 170.9, 165.4, 149.7, 146.5, 137.4, 134.8, 133.6, 133.5, 132.2, 130.7, 127.9, 126.7, 126.5, 125.56, 125.4, 125.0, 121.4, 120.4, 118.0, 114.9, 54.8, 52.4, 51.0, 50.5, 41.2, 37.4, 37.0, 24.8, 22.6, 22.0, 20.8. ^{31}P NMR (121 MHz, CDCl$_3$) δ = -2.5, -2.6. MS (ES) m/z 751.4 [M+Na]$^+$. HRMS C$_{38}$H$_{41}$N$_4$O$_9$NaP calcd. 751.2509 [M+Na]$^+$, found 751.2513.

Methyl ((2*S*)-2-(4-cyanobenzamido)-3-(4-(((((*S*)-1-isopropoxy-1-oxopropan-2-yl)amino)(naphthalen-1-yloxy)phosphoryl)oxy)phenyl)propanoyl)-*L*-leucinate (14) In a flame dried flask, a solution of compound **5** (70 mg, 0.160 mmol) in DCM (5 mL) was stirred with triethylamine (0.05 mL, 0.352 mmol) at room temperature for 15 minutes under an argon atmosphere. Compound **11** (252 mg, 0.708 mmol) was added dropwise in DCM (3 mL). The yellow solution was stirred at room temperature for 5 hours; where it was then concentrated under reduced pressure to form a dense yellow oil. The oil was diluted in water (20 mL) and the product was extracted with ethyl acetate (3x20 mL), where the yellow oil was washed with water (20 mL) and brine (20 mL), dried with MgSO$_4$, filtered and concentrated to form a white solid. Column chromatography (2:1 ethyl acetate: hexane) was used to isolate prodrug **14** as white crystals (110 mg, 91%). ^{1}H NMR (400 MHz, CDCl$_3$) δ = 8.01 (dd, J = 8.0, 5.3 Hz, 1H), 7.87 – 7.80 (m, 1H), 7.74 (dd, J = 9.8, 8.5 Hz, 2H), 7.65 (d, J = 8.3 Hz, 1H), 7.57 (d, J = 8.5 Hz, 1H), 7.53 (t, J = 3.1 Hz, 1H), 7.52 – 7.44 (m, 3H), 7.41 – 7.35 (m, 2H), 7.23-7.12 (m, 4H), 6.64 (dd, J = 46.4, 8.1 Hz, 1H), 5.04 – 4.78 (m, 2H), 4.62 – 4.50 (m, 1H), 4.23 (dd, J = 12.3, 9.8 Hz, 1H), 4.17 – 4.07 (m, 1H), 3.68 (d, J = 9.2 Hz, 3H), 3.13 (t, J = 5.8 Hz, 2H), 1.65 – 1.43 (m, 3H), 1.38 – 1.31 (m, 3H), 1.25 (dd, J = 17.7, 7.3 Hz, 1H), 1.20 – 1.06 (m, 6H), 0.83 (d, J = 2.8 Hz, 6H). ^{13}C NMR (101 MHz, CDCl$_3$) δ = 172.8, 170.5, 165.3, 149.9, 146.6, 137.5, 134.8, 133.6, 133.2, 132.3, 130.8, 127.8, 126.6, 126.4, 125.5, 125.0, 121.4, 120.5, 118.0, 115.2, 115.0, 69.4, 54.8, 52.4, 51.0, 50.6, 41.3, 37.5, 37.1, 24.8, 22.6, 21.8, 21.6, 21.0. ^{31}P NMR (121 MHz, CDCl$_3$) δ = -2.5, -2.5. MS (ES) m/z 757.3 [M+H]$^+$, 779.3 [M+Na]$^+$. HRMS C$_{40}$H$_{45}$N$_4$O$_9$NaP calcd. 779.2822 [M+Na]$^+$, found 779.2825.

Methyl ((2*S*)-3-(4-(((((*S*)-1-(benzyloxy)-1-oxopropan-2-yl)amino)(naphthalen-1-yloxy)phosphoryl)oxy)phenyl)-2-(4-cyanobenzamido)propanoyl)-*L*-leucinate (15) In a flame dried flask, a solution of compound **5** (70 mg, 0.160 mmol) in DCM (5 mL) was stirred with triethylamine (0.05 mL, 0.352 mmol) at room temperature for 15 minutes under an argon atmosphere. Compound **12** (350 mg, 0.867 mmol) was added dropwise in DCM (3 mL). The yellow solution was stirred at room temperature for 5 hours; where it was then concentrated under reduced pressure to form a dense yellow oil. The oil was diluted in water (20 mL) and the product was extracted with ethyl acetate (3x20 mL), where the yellow oil was washed with water (20 mL) and brine (20 mL), dried with MgSO$_4$, filtered and concentrated to form a white solid. Column chromatography (2:1 ethyl acetate: hexane) was used to isolate prodrug **15** as white crystals (120 mg, 93%). ^{1}H NMR (400 MHz, CDCl$_3$)

δ = 7.99 (t, J = 7.8 Hz, 1H), 7.83 (t, J = 8.3 Hz, 1H), 7.72 (dd, J = 11.7, 8.5 Hz, 2H), 7.67 – 7.59 (m, 1H), 7.53 (d, J = 8.5 Hz, 2H), 7.50 – 7.39 (m, 3H), 7.38 – 7.31 (m, 1H), 7.30 – 7.19 (m, 5H), 7.18 – 7.04 (m, 4H), 6.81 (dd, J = 59.9, 8.1 Hz, 1H), 5.00 (q, J = 12.2 Hz, 2H), 4.92 – 4.80 (m, 2H), 4.56 (dd, J = 8.5, 4.4 Hz, 1H), 4.39 (dd, J = 12.4, 10.0 Hz, 1H), 4.30 – 4.12 (m, 1H), 3.66 (d, J = 7.0 Hz, 3H), 3.19 – 3.02 (m, 2H), 1.65 – 1.45 (m, 3H), 1.37 (dd, J = 22.5, 7.1 Hz, 3H), 1.29 – 1.23 (m, 1H), 0.83 (d, J = 5.6 Hz, 6H). ^{13}C NMR (101 MHz, CDCl$_3$) δ = 172.9, 170.7, 165.3, 149.8, 146.5, 137.5, 135.1, 134.7, 133.4, 132.3, 130.8, 128.6, 128.5, 128.1, 127.8, 126.7, 126.6, 126.4, 125.5, 125.4, 125.0, 121.4, 120.4, 118.0, 115.1, 67.2, 54.9, 52.4, 51.0, 50.5, 41.2, 37.4, 36.9, 24.8, 22.6, 21.8, 20.9. ^{31}P NMR (121 MHz, CDCl$_3$) δ = -2.5, -2.6. MS (ES) m/z 827.3 [M+Na]$^+$. HRMS C$_{44}$H$_{45}$N$_4$O$_9$NaP calcd. 827.2822 [M+Na]$^+$, found 827.2826.

Serum stability of prodrug 14. Procedure was adapted from Slusarczyk et al.[15] Compound **14** (5mg) was dissolved in DMSO (0.15 mL) and D$_2$O (0.15 mL) in an NMR tube. A ^{31}P-NMR of the sample was initially undertaken as a control. Defrosted human serum (0.3 mL) was added to the NMR tube, which initially formed a cloudy solution. Additional DMSO (0.15 mL) was treated to aid solubility. The tube was then incubated at 37 $^\circ$C and the spectra were recorded at 30 min after the addition and then at even time intervals over 12 hr. At the end of the ^{31}P-NMR experiment, the organics were extracted from the serum with ethyl acetate (3x1 mL), dried with MgSO$_4$, filtered and concentrated under reduced pressure to yield a white solid. A ^{31}P-NMR of this in DMSO (0.15 mL) and D$_2$O (0.15 mL) was taken and this demonstrated the retention of the prodrug singlet pair. The extract was additionally used for LCMS.

Cell Assay and Immunoblotting. MDA-MB-468 cells were cultured in RPMI 1640 media (Gibco) supplemented with 10% Fetal Bovine Serum (FBS) (Gibco) and 1% of PenStrep (Gibco). The cell was maintained at 37 °C with 5% CO$_2$ incubator to reach 70-80% confluency to be used in the experiment. The cells were treated with ISS-610, ISS-610-Met, or prodrugs **13**-**15** in various concentrations (0.1, 1, 10, 30 and 100 μM) for 24 hours. Protein lysates were prepared using 1% NP40 lysis buffer containing 50mM Tris-HCl pH 7.5, 1mM EDTA, 1mM EGTA, 1% Nonidet-40, 0.27 M Sucrose, 1 mM sodium orthovanadate, 50 mM sodium fluoride, 5 mM sodium pyrophosphate supplemented with 1 mM benzamidine and 0.1 mM PMSF. Protein concentrations were measured using Bradford assay and SDS samples were prepared by boiling the protein sample at 90 °C for 5 minutes in SDS sample loading buffer. 20 μg of each sample was loaded per lane and subjected to separation in polyacrylamide gels and transfer on to nitrocellulose membrane. Membranes were blocked in 10% skim milk in TBST [50 mM Tris/HCl (pH 7.5), 0.15 M NaCl containing 0.25% Tween-20] for 30 minutes at room temperature. The membranes were then incubated with the primary antibodies STAT3 (Cell Signalling Technology, #4904), Survivin (Cell Signalling Technology, #2808), Bcl-xl (Cell Signalling Technology, #2764) and β-actin (Cell Signalling Technology, #3700), overnight at 4 °C and secondary antibodies: anti-rabbit (Cell Signalling Technology, #7074) or anti-mouse (Cell Signalling Technology, #7076), for 1 hr at room temperature. Finally, protein detection using horseradish peroxidase-conjugated secondary antibodies and the ECL® reagent (Amersham Bioscience) was performed.

Conflicts of interest

The authors declare no conflicts of interest.

Author Contributions

A.M. synthesized and characterized the compounds presented in this work. B.A.D. executed the biological characterization of the compounds. A.M., B.A.D. and Y.M. designed all of the experiments. Y.M. wrote the manuscript and all of the authors provided feedback and approved the final version of the manuscript.

ABBREVIATIONS

POCl$_3$, phosphorous (V) oxychloride; SH$_2$, Src homology 2; STAT3, Signal transducer and activator of transcription 3; TEA, triethylamine; TFA, trifluoroacetic acid.

Notes and references

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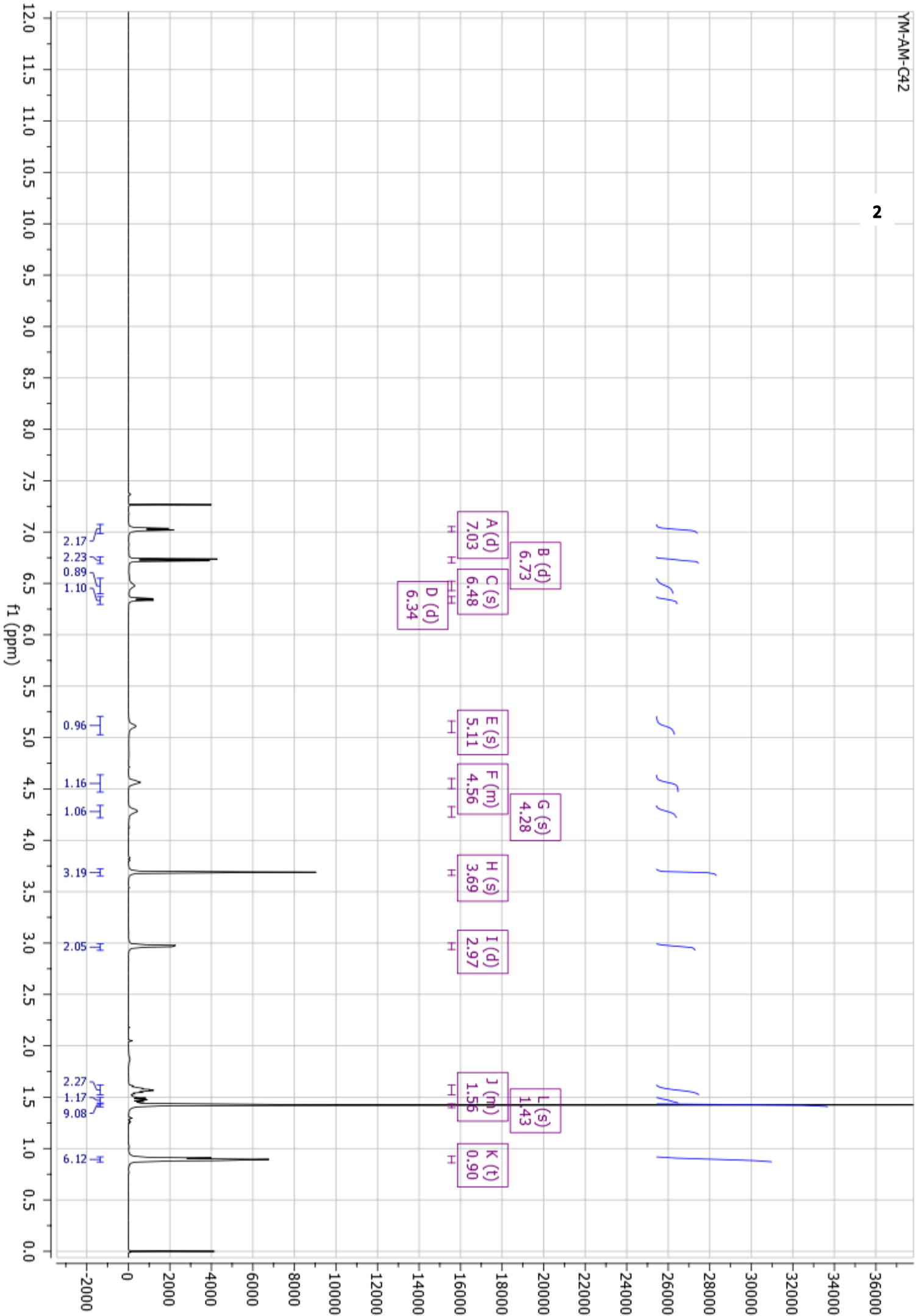
SUPPORTING INFORMATION

Phosphotyrosine Prodrugs: Design, Synthesis and Anti-STAT3 Activity of ISS-610 Aryloxy Triester Phosphoramidate Prodrugs

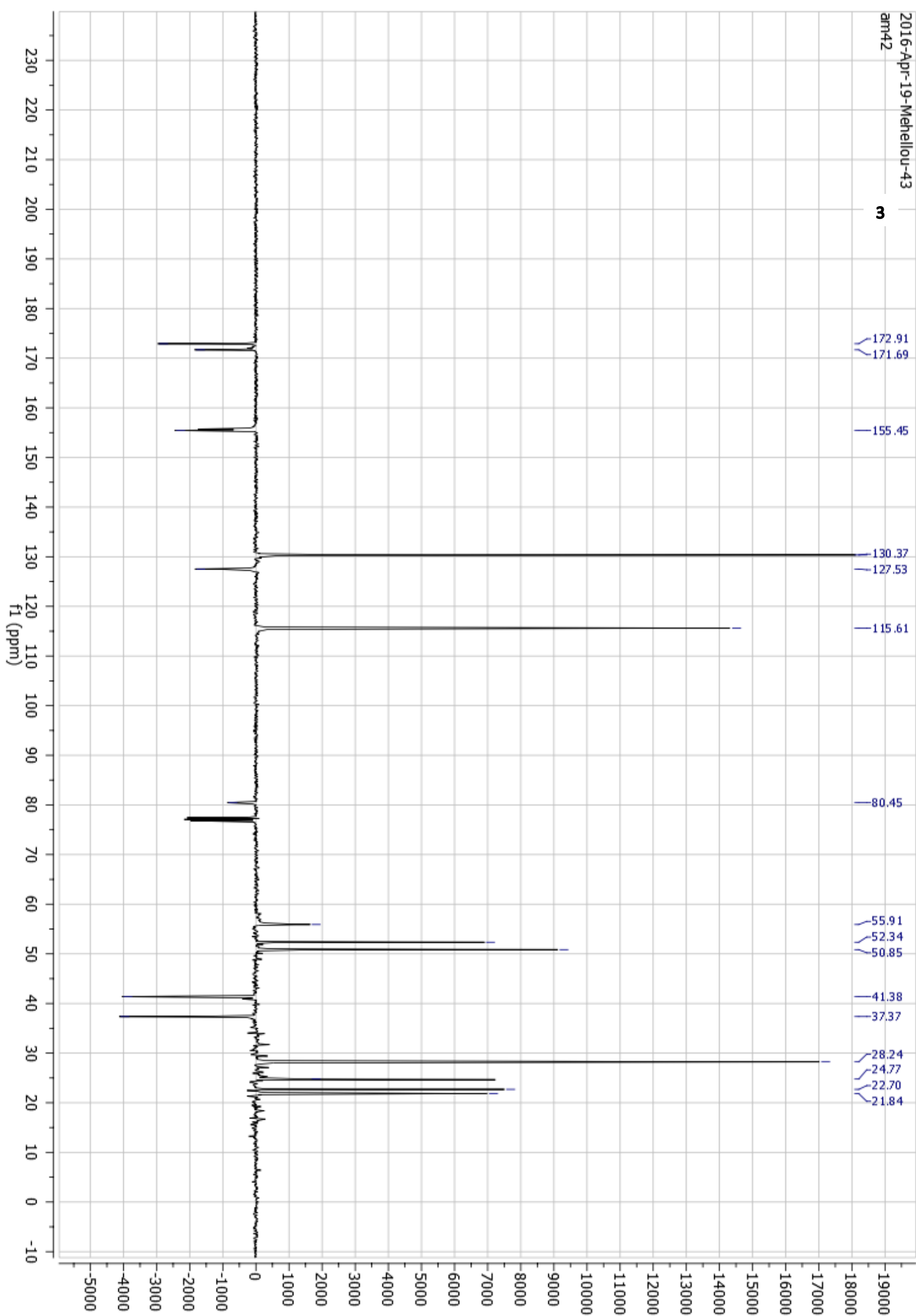Ageo Miccoli,[a] Binar A. Dhiani, [a] and Youcef Mehellou*[a]

[a]School of Pharmacy and Pharmaceutical Sciences. Redwood Building, Cardiff University, Cardiff, CF10 3NB, UK.
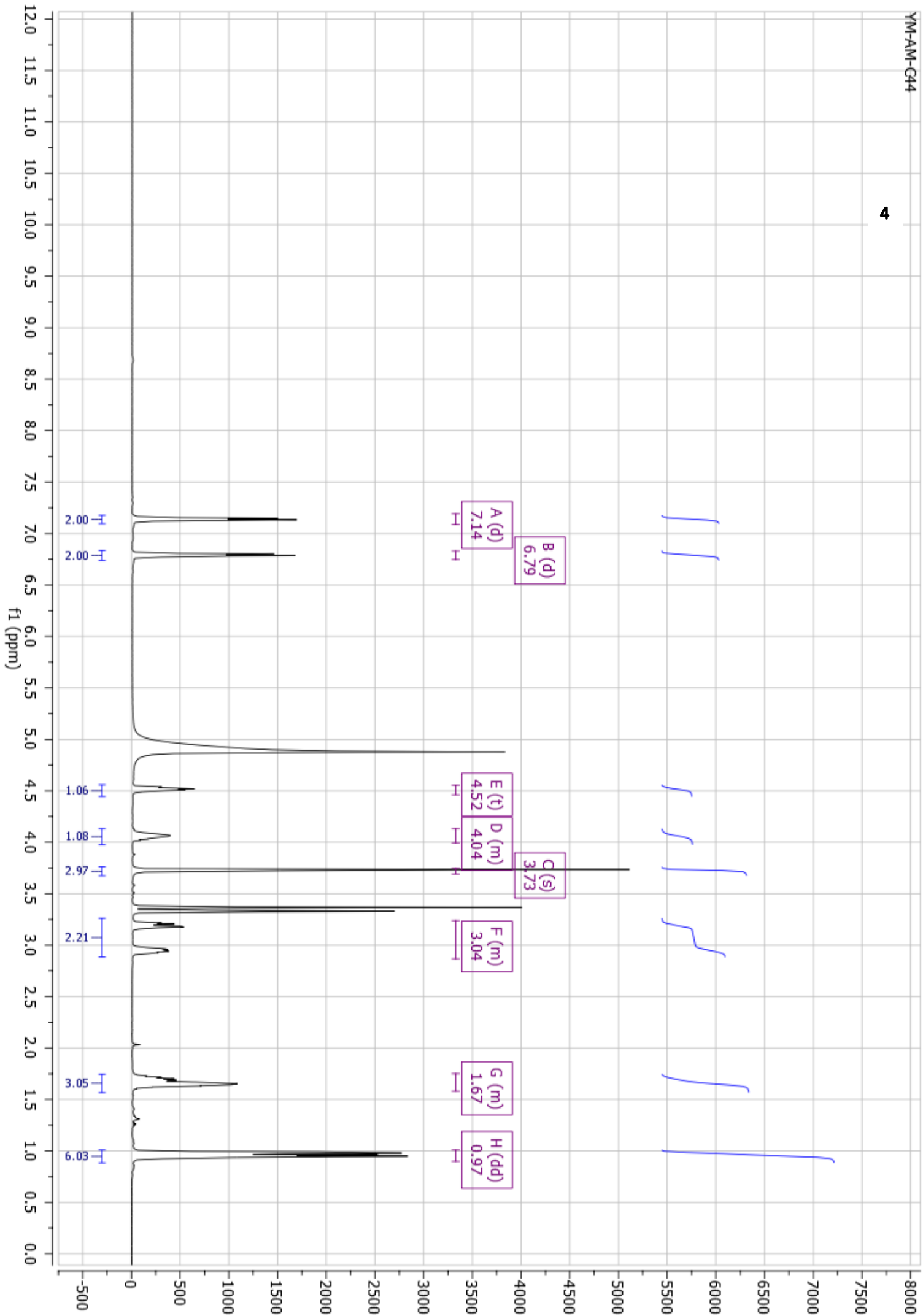
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4

5

f1 (ppm)

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155.35
137.37
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37.83
24.80
22.63
21.85

Peak assignments:

Label	Multiplicity	Shift (ppm)
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F	d	7.65
G	d	7.62
H	d	7.18
I	d	7.06
J	d	7.02
K	q	4.89
L	td	4.55
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C	ddd	2.69
M	m	1.61
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D	dd	0.87

Integrations: 1.97, 0.95, 1.92, 1.99, 1.89, 0.77, 1.00, 1.00, 2.87, 2.00, 11.67, 2.12, 1.24, 5.88

9

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f1 (ppm)

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15.79

f1 (ppm)

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f1 (ppm)

14

Chromatogram Report

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15

16

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7.85

f1 (ppm)

Please do not adjust margins

18

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f1 (ppm)

20

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22

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f1 (ppm)

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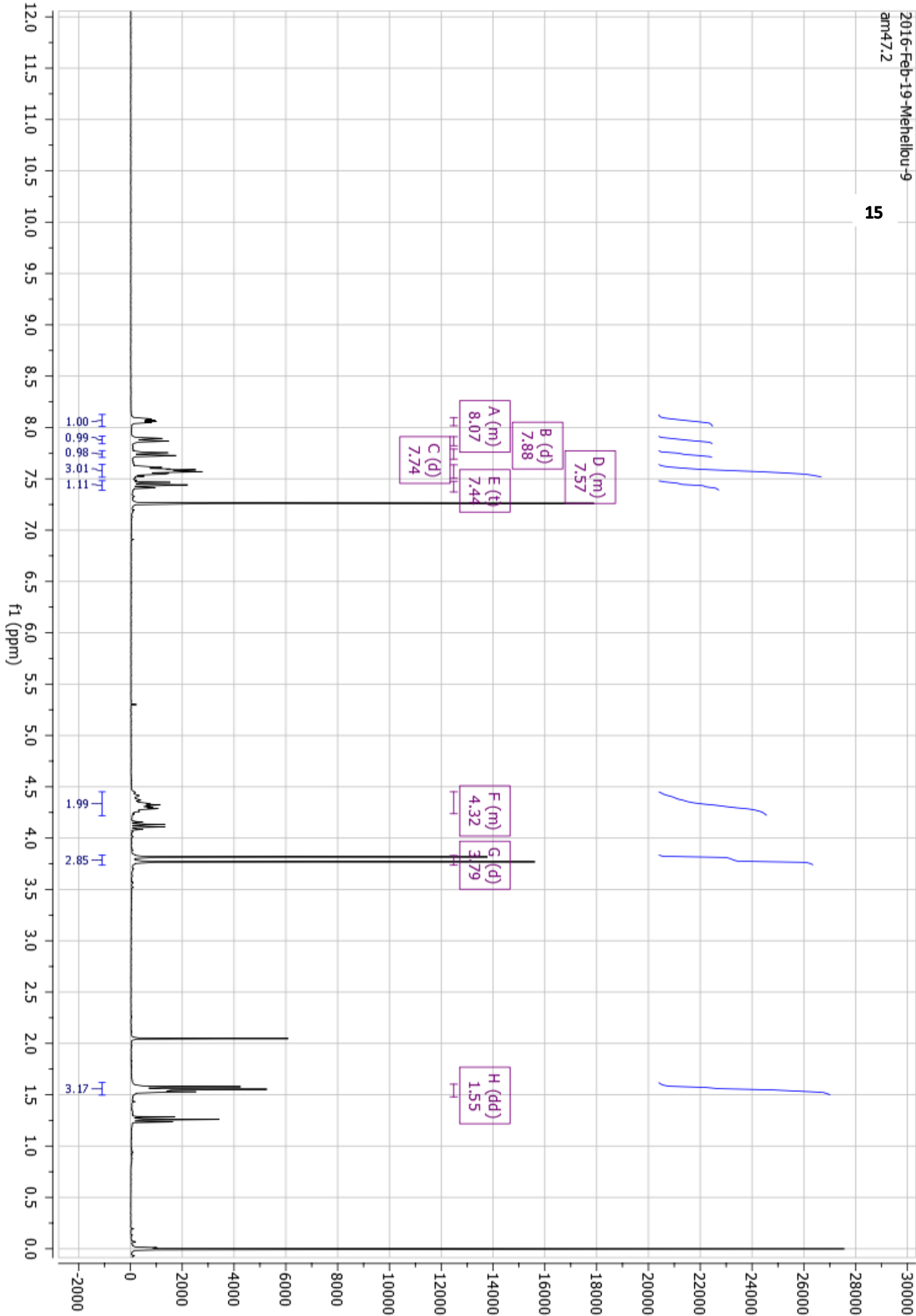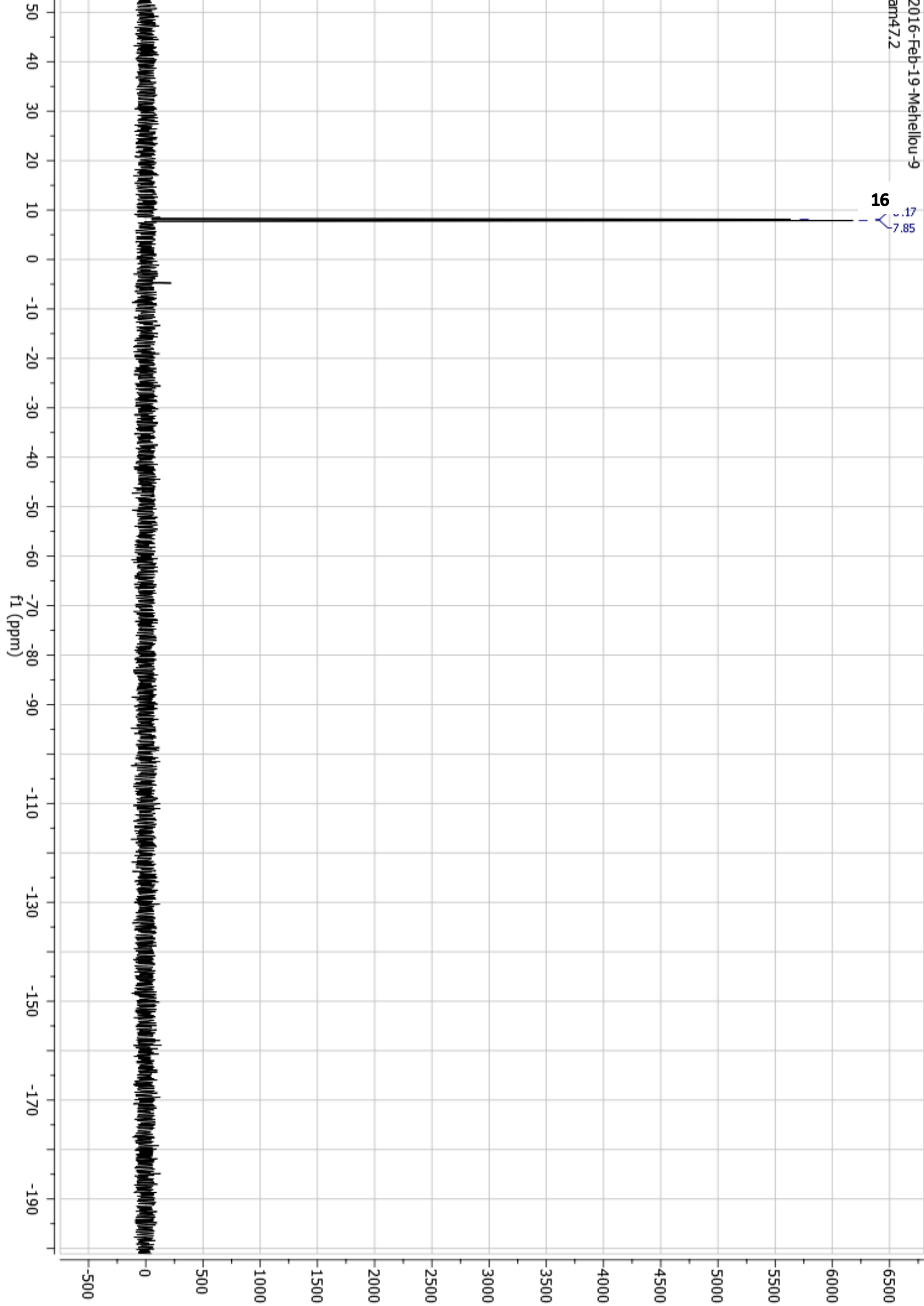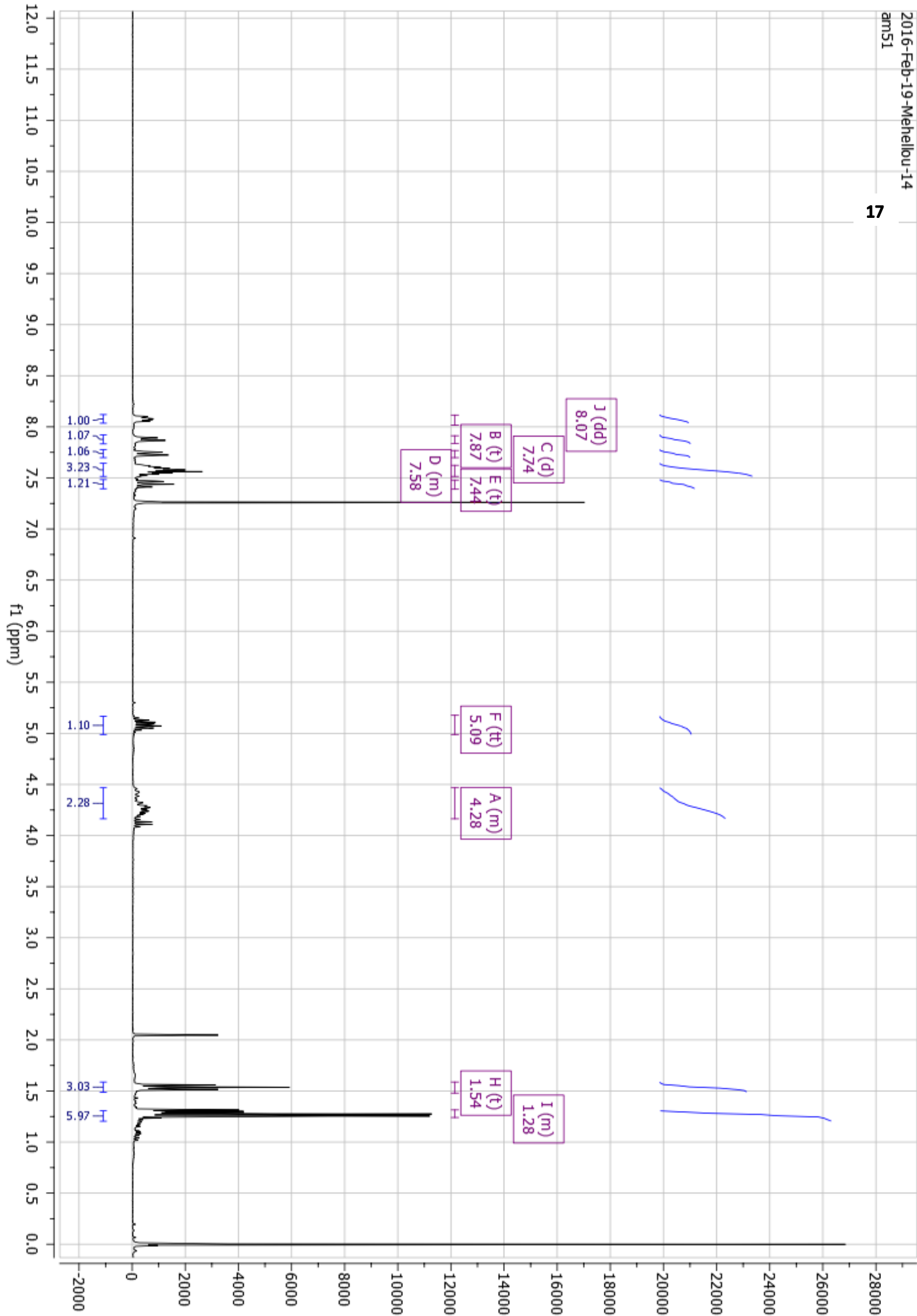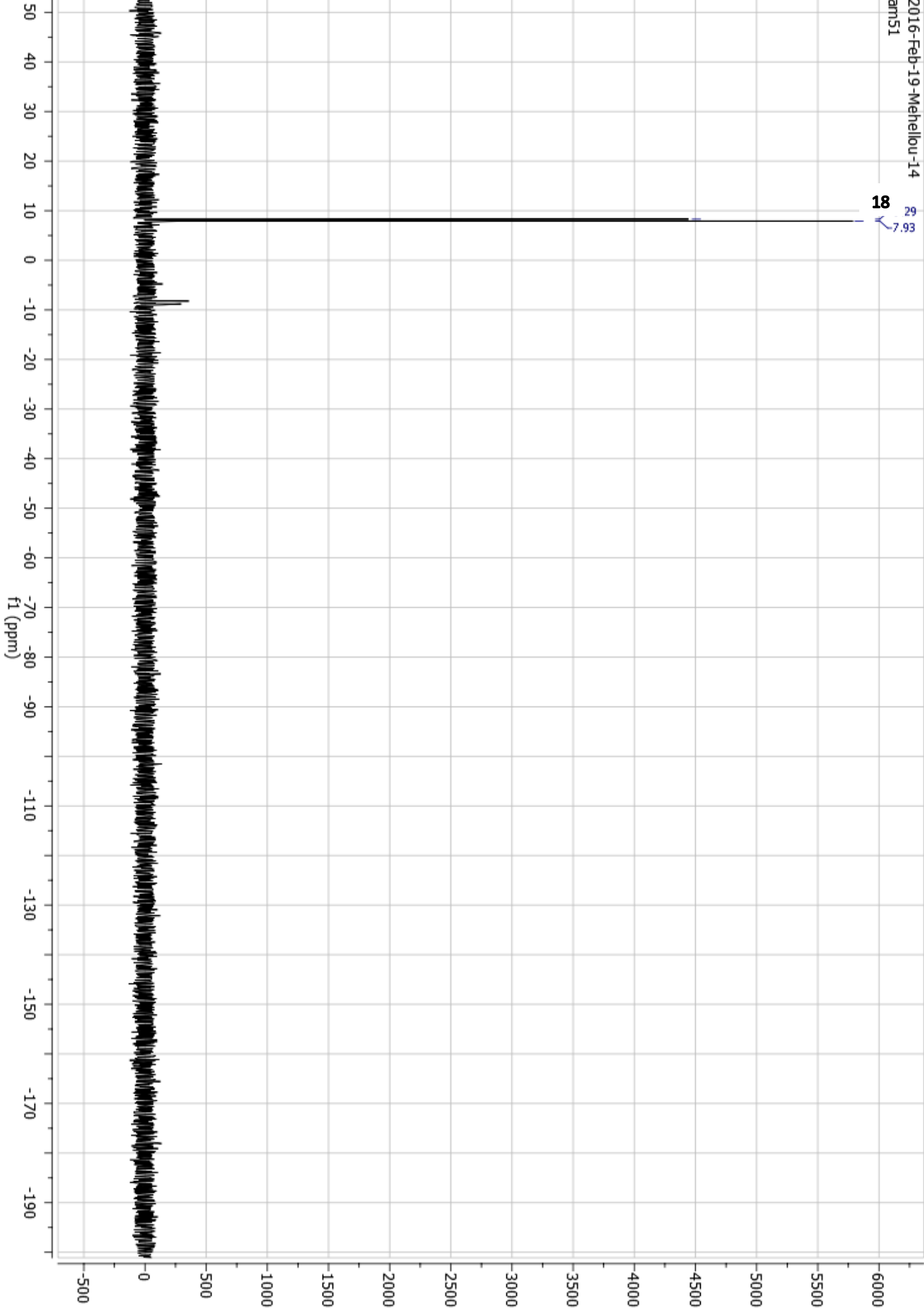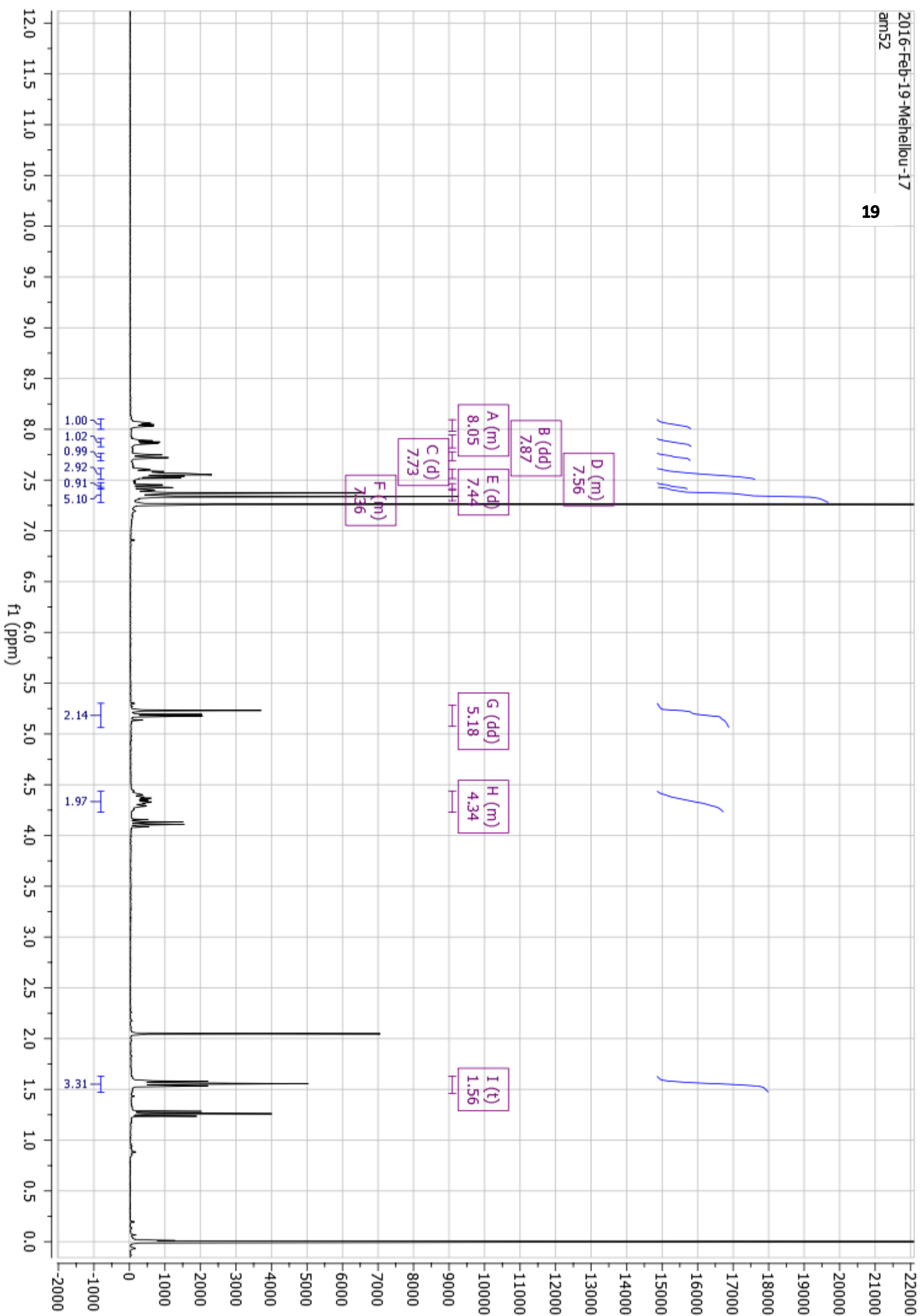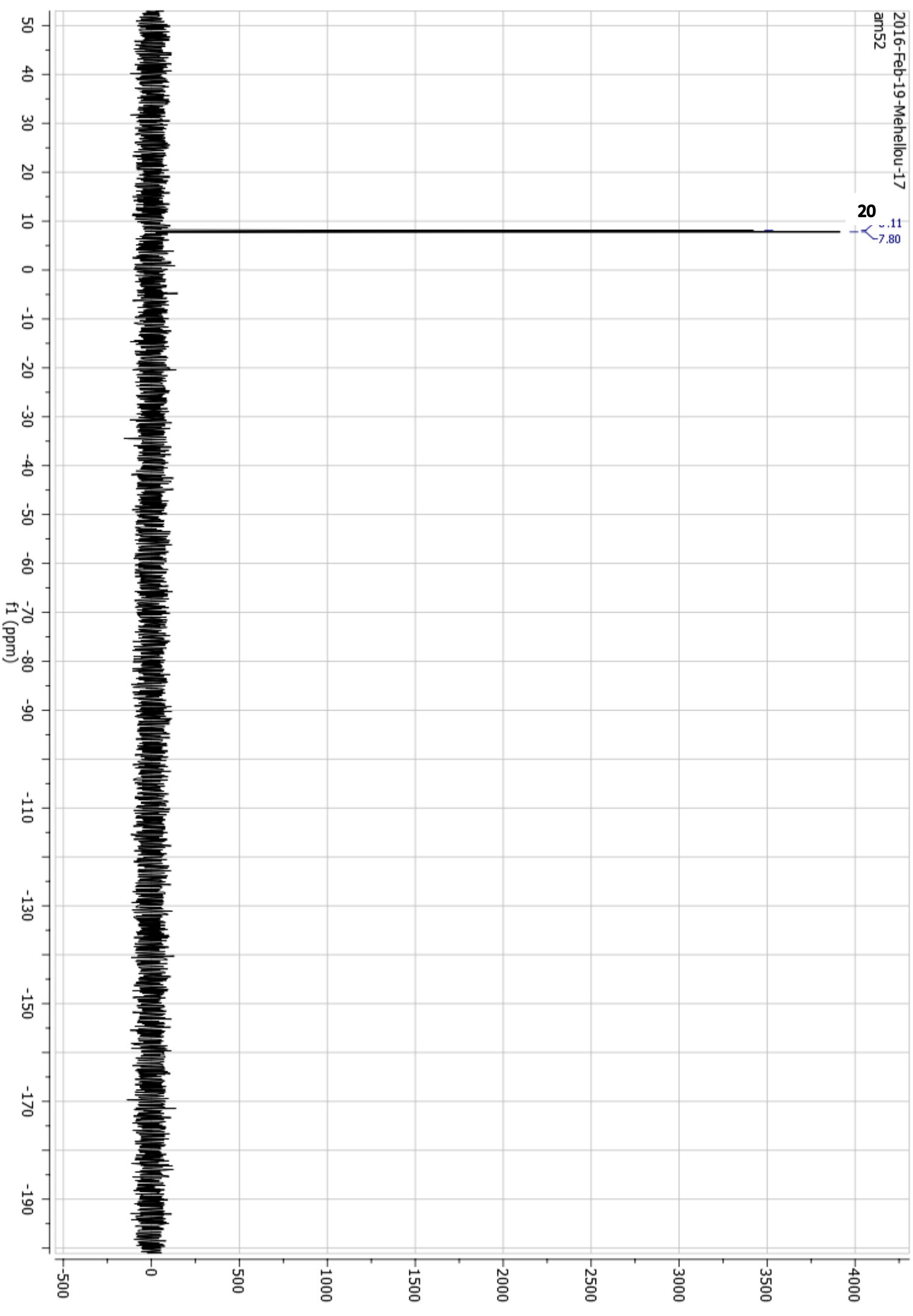
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Method          : C:\Chem32\1\Data\AllenB\AAB 30_06_16 AM 2016-06-30 09-28-02\AB Gradient
                  0to100 C=MeCN D=H2O.M (Sequence Method)
Last changed    : 6/30/2016 9:28:02 AM by SYSTEM
Additional Info : Peak(s) manually integrated
```
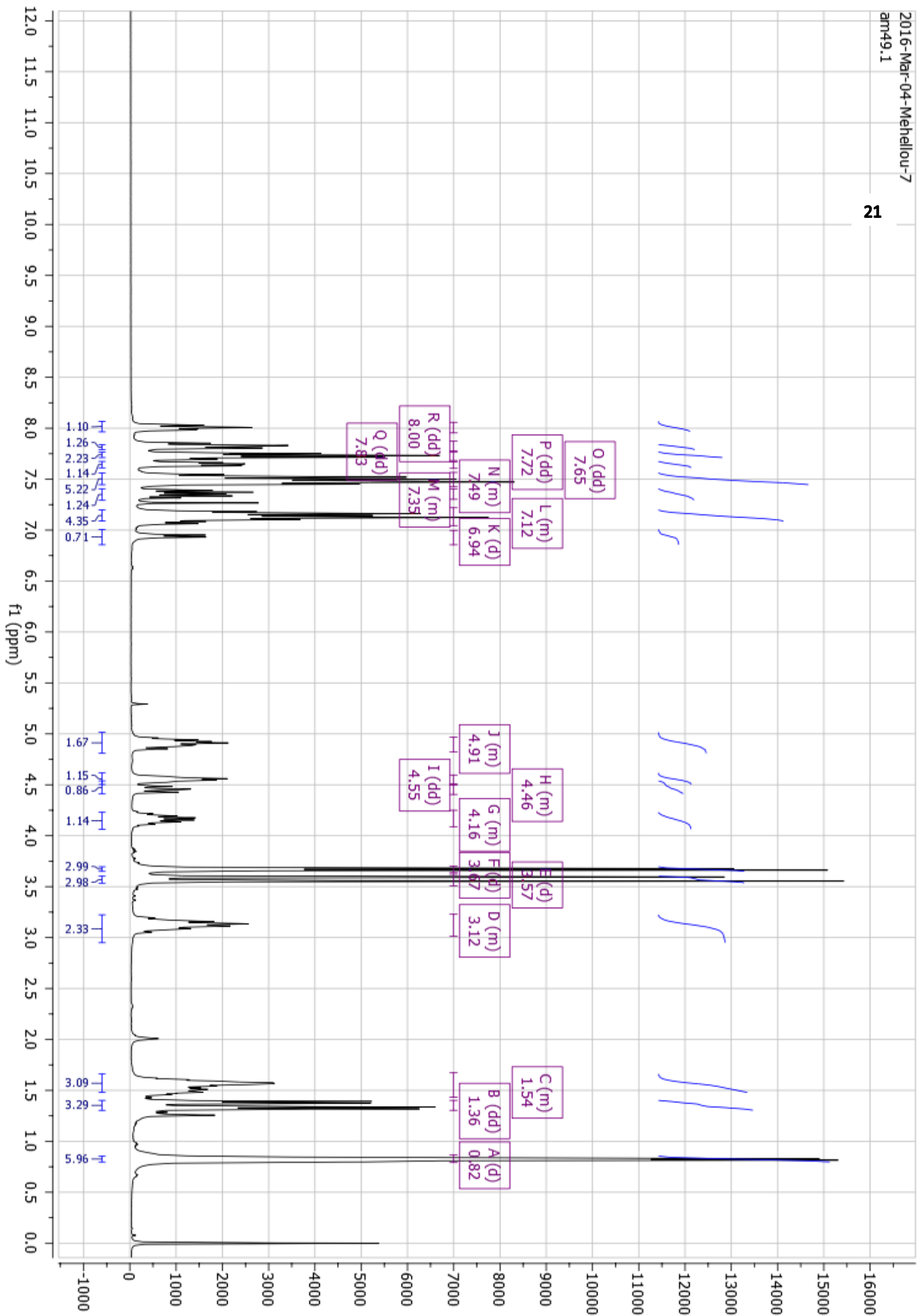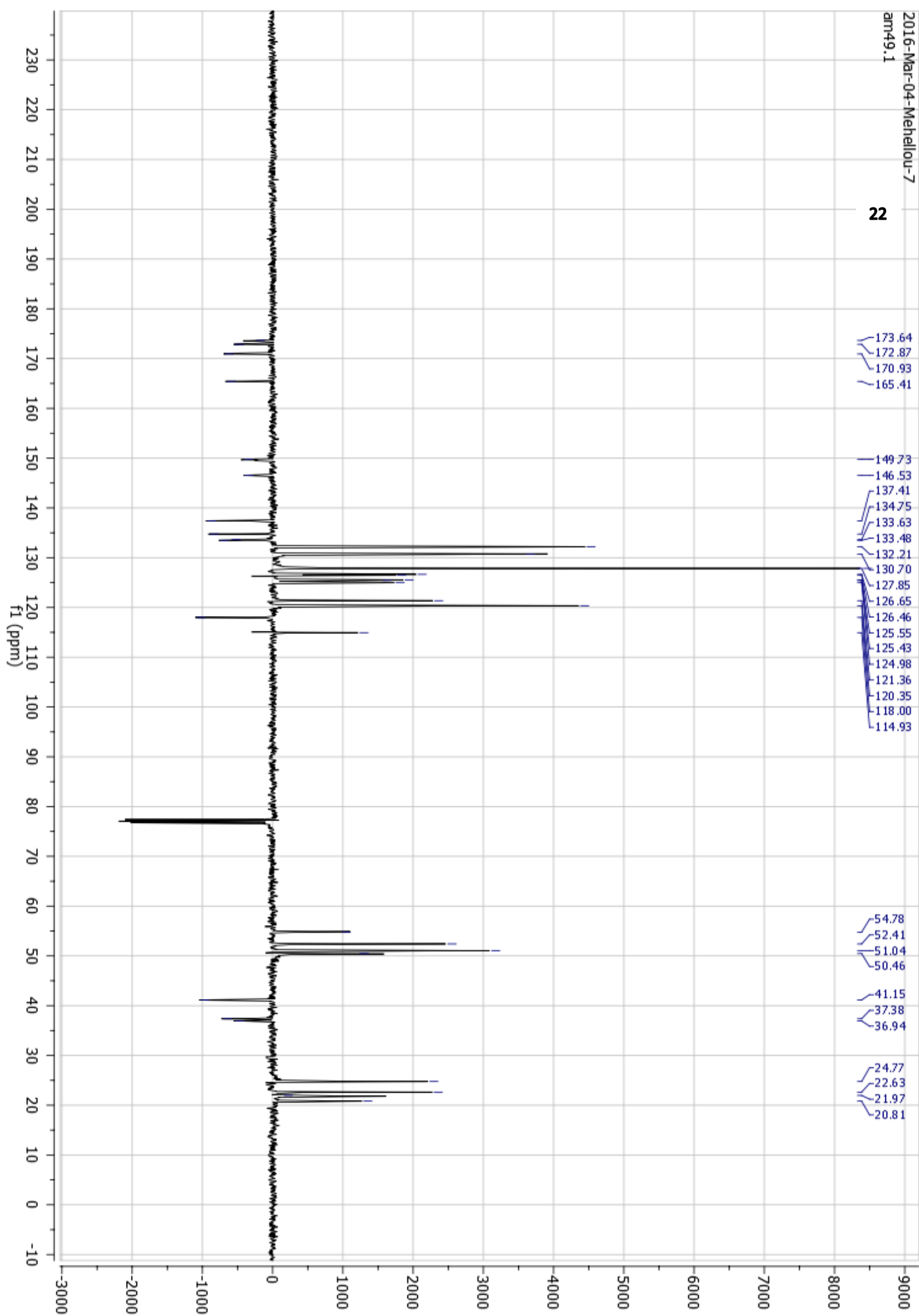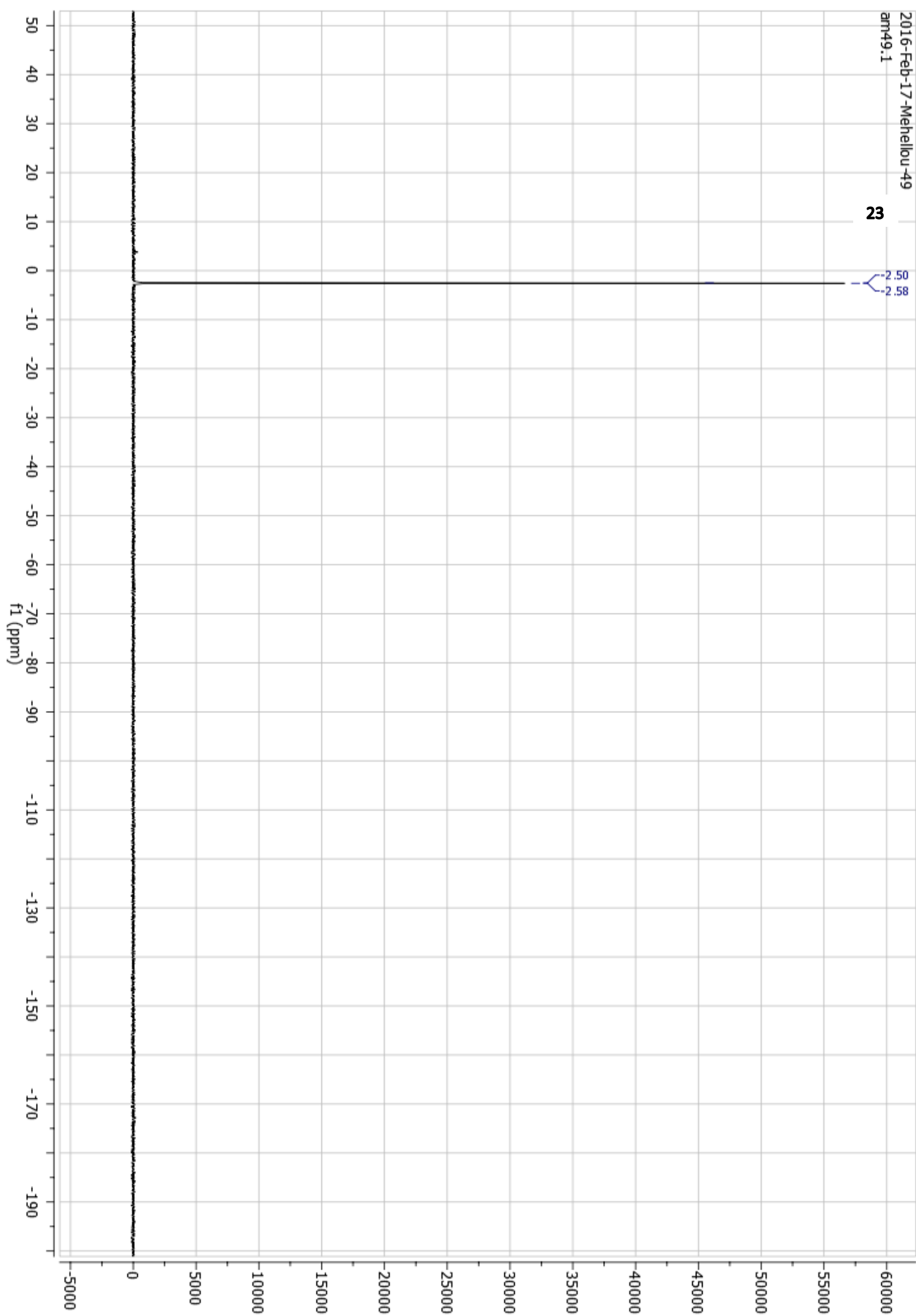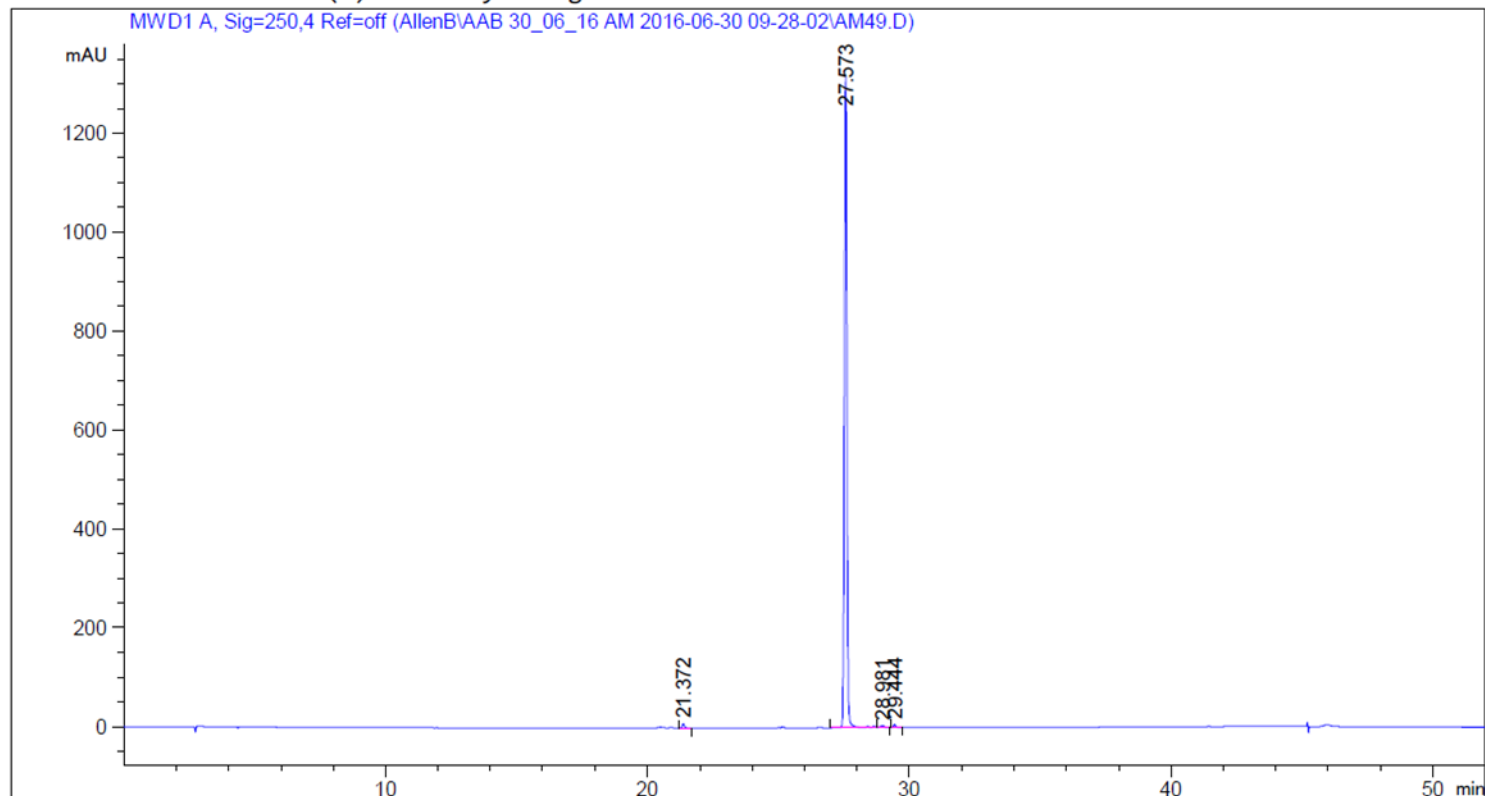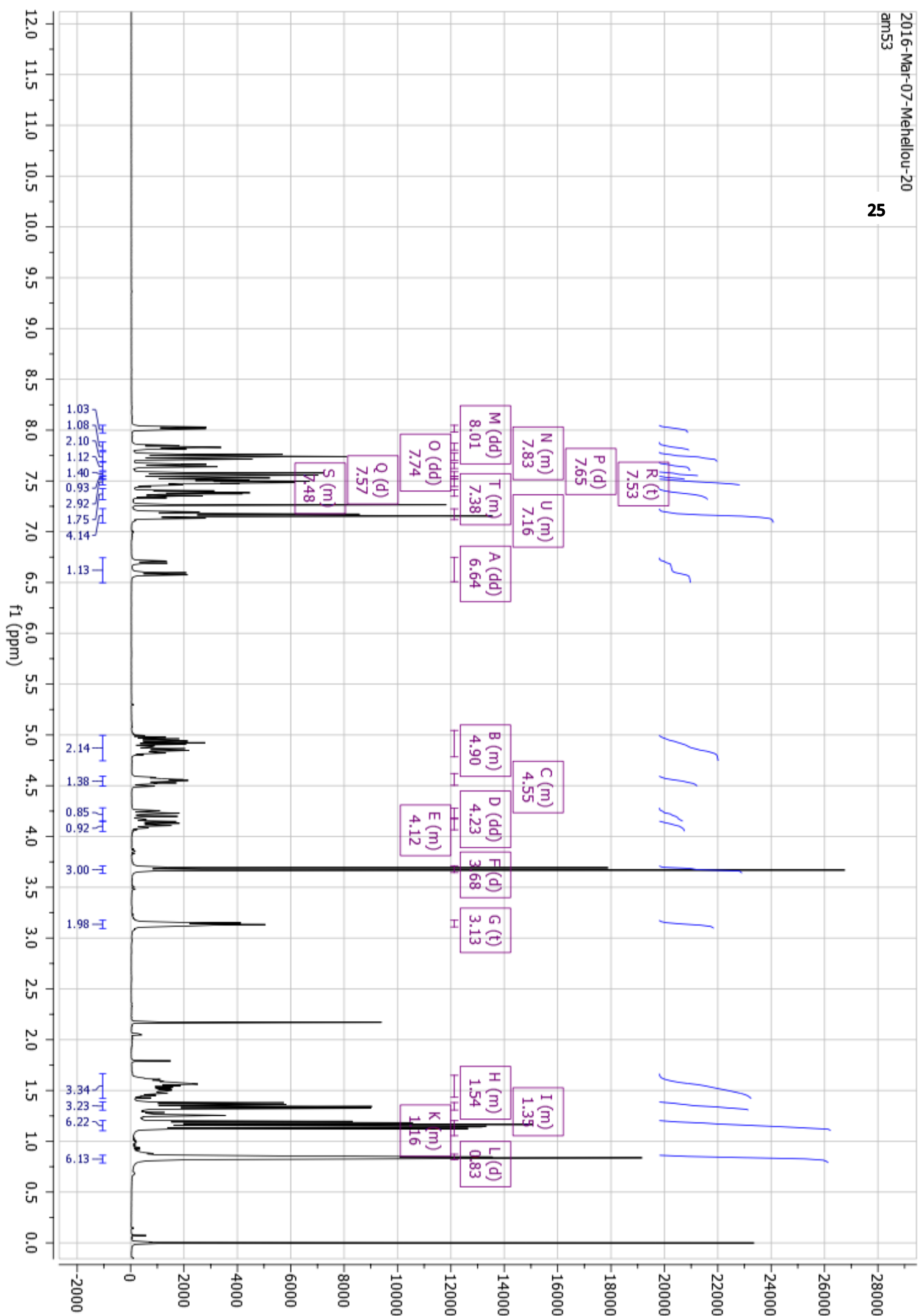
24


```
===================================================================
                     Area Percent Report
===================================================================


Sorted By          :        Signal
Multiplier         :        1.0000
Dilution           :        1.0000
Use Multiplier & Dilution Factor with ISTDs



Signal 1: MWD1 A, Sig=250,4 Ref=off

Peak RetTime Type  Width     Area        Height     Area
 #    [min]        [min]   [mAU*s]       [mAU]       %
----|-------|----|-------|----------|----------|--------|
 1   21.372 BB   0.0846   48.79847    8.81579   0.5482
 2   27.573 VV R 0.1053 8772.26758 1317.55127  98.5478
 3   28.981 VB E 0.1521   43.55955    4.16072   0.4893
 4   29.444 BB   0.0882   36.90587    6.31590   0.4146

Totals :                8901.53146 1336.84368


===================================================================
```

26

172.80
170.47
165.32

149.88
146.55
137.51
134.76
133.55
133.22
132.32
130.77
127.79
126.64
126.37
125.54
124.96
121.44
120.45
117.97
115.18
115.02

69.35

54.81
52.38
51.02
50.62

41.27
37.49
37.06

24.78
22.64
21.83
21.58
20.98

-2.48
-2.52

f1 (ppm)

```
=================================================================
Acq. Operator   : SYSTEM                    Seq. Line :   4
Acq. Instrument : LC                        Location :   4
Injection Date  : 6/30/2016 12:07:53 PM          Inj :   1
                                          Inj Volume : 10.000 µl
Method          : C:\Chem32\1\Data\AllenB\AAB 30_06_16 AM 2016-06-30 09-28-02\AB Gradient
                  0to100 C=MeCN D=H2O.M (Sequence Method)
Last changed    : 6/30/2016 9:28:02 AM by SYSTEM
Additional Info : Peak(s) manually integrated
```

28


```
=================================================================
                    Area Percent Report
=================================================================

Sorted By           :      Signal
Multiplier          :      1.0000
Dilution            :      1.0000
Use Multiplier & Dilution Factor with ISTDs


Signal 1: MWD1 A, Sig=250,4 Ref=off

Peak RetTime Type  Width     Area       Height     Area
  #   [min]        [min]   [mAU*s]      [mAU]       %
----|-------|----|-------|----------|----------|--------|
  1  29.530 VV R  0.1379 1.08418e4   1291.95044  99.2065
  2  30.493 VB E  0.1219   46.54359     6.03982   0.4259
  3  30.903 BB    0.1225   40.17849     4.66466   0.3676

Totals :                 1.09285e4   1302.65491


=================================================================
                    *** End of Report ***
```
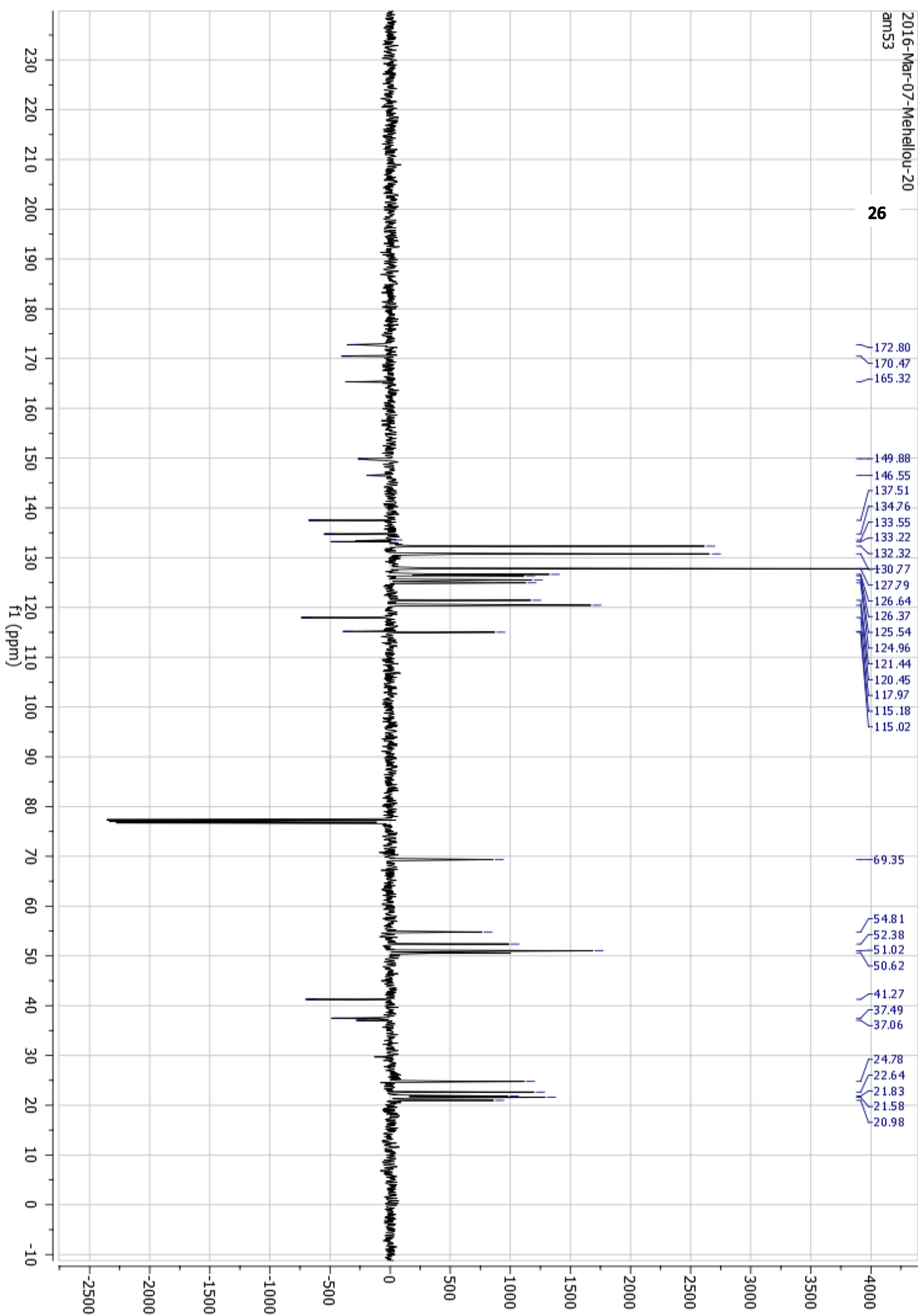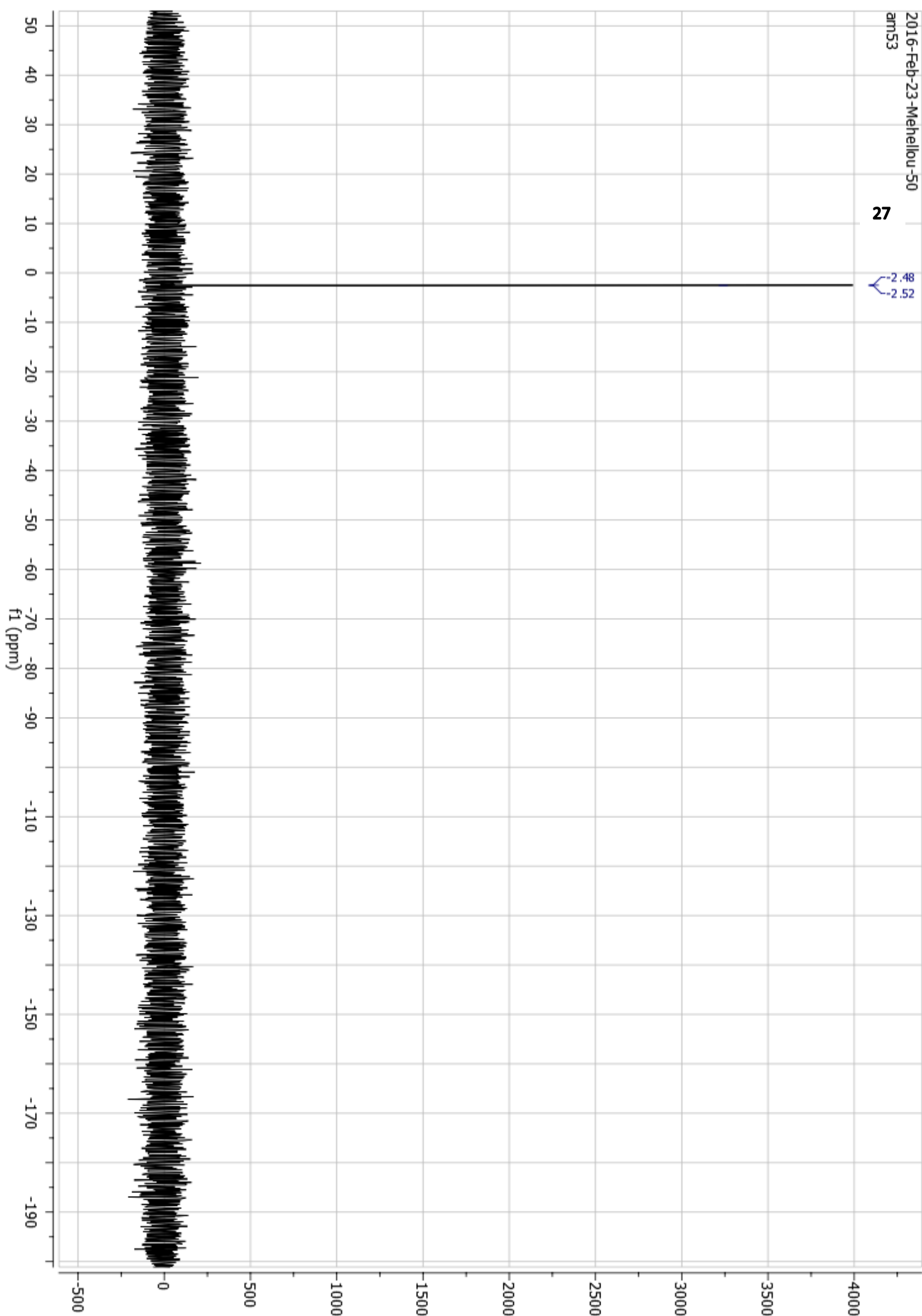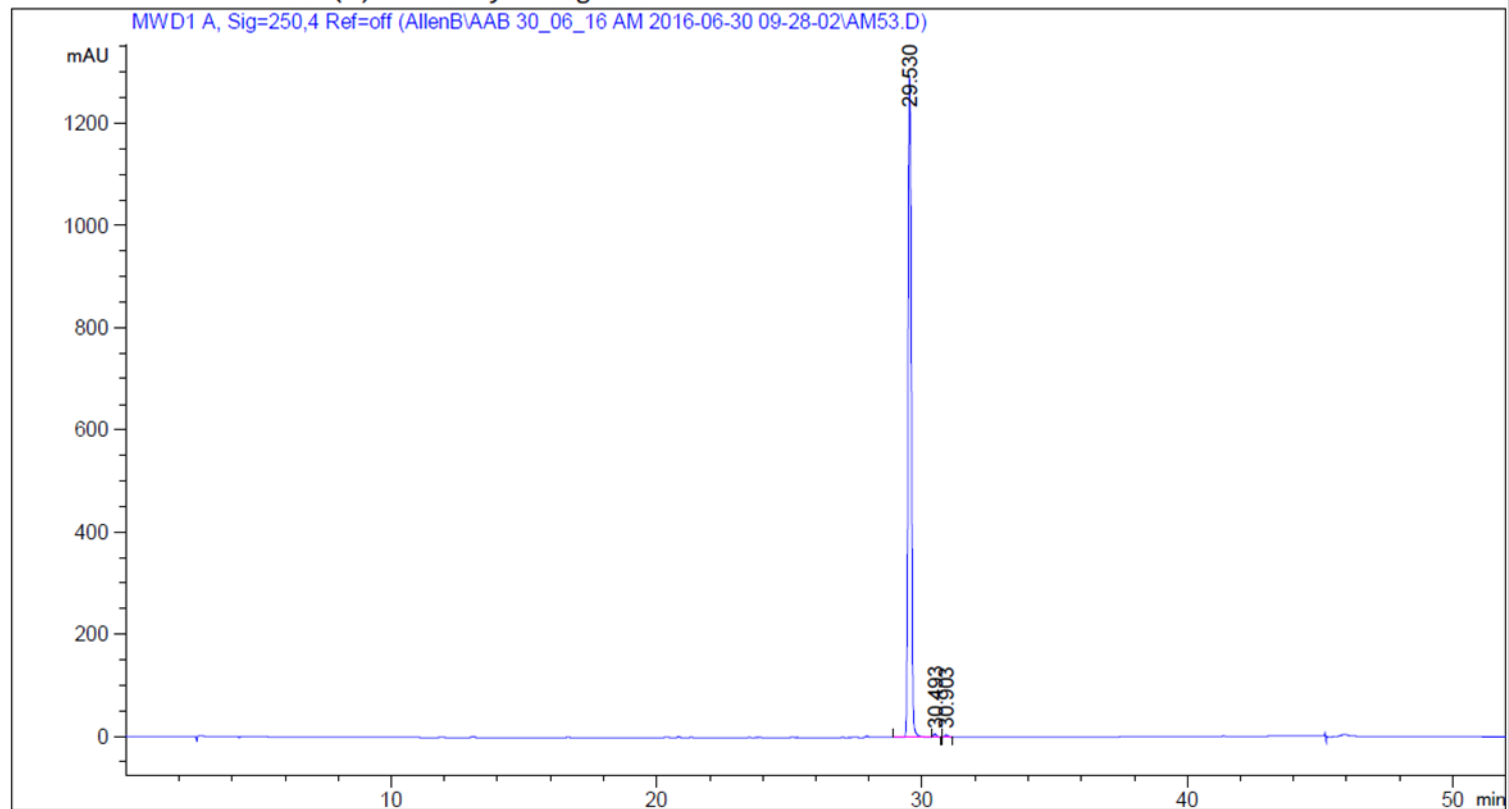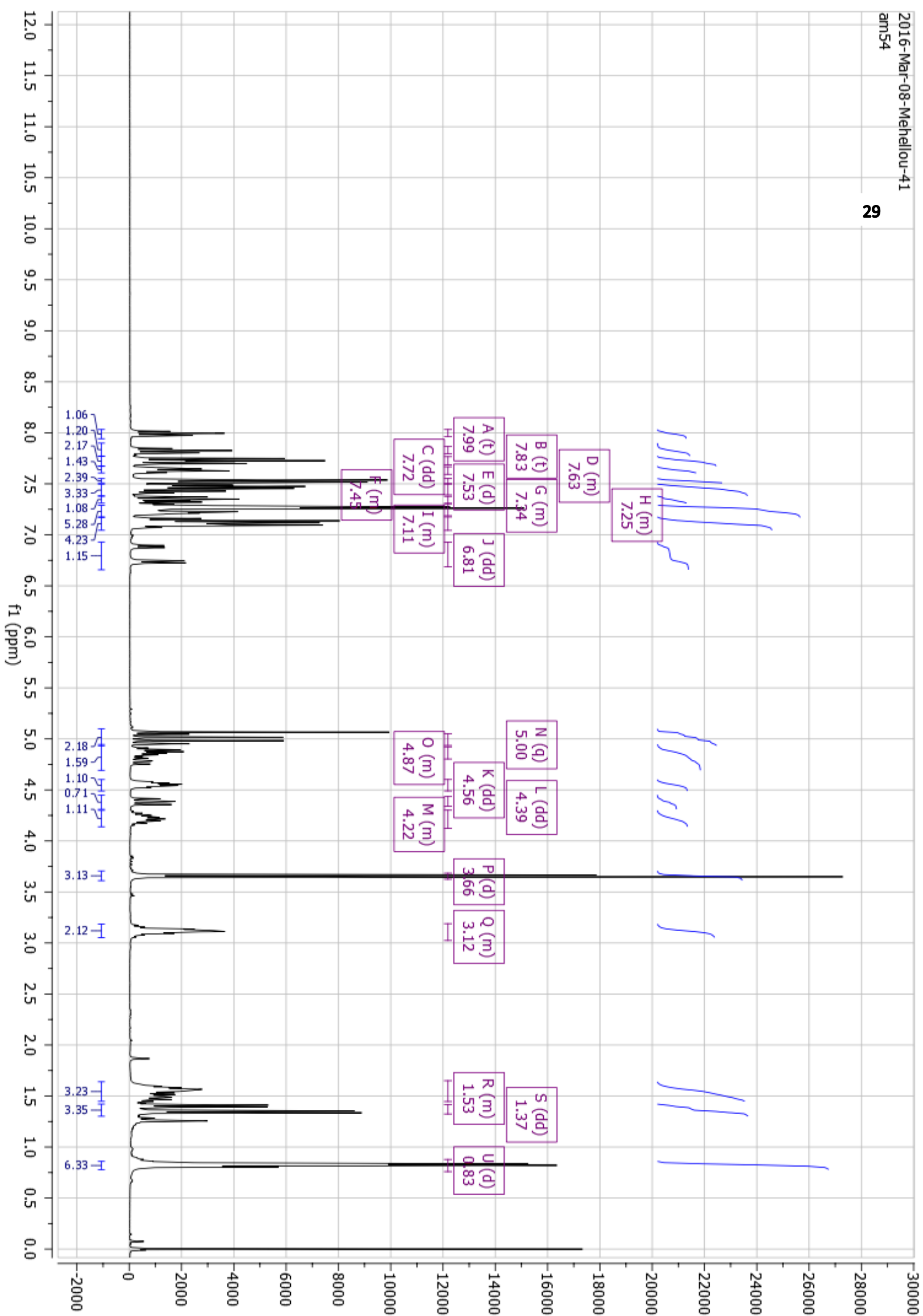
30

172.89
170.70
165.26

149.77
146.51
137.46
135.10
134.74
133.36
132.27
130.77
128.60
128.47
128.13
127.82
126.72
126.64
126.41
125.54
125.44
125.00
121.40
120.40
117.99
115.06

67.24

54.85
52.36
51.02
50.52

41.19
37.42
36.93

24.77
22.64
21.80
20.93

f1 (ppm)

31

-2.48
-2.60

f1 (ppm)
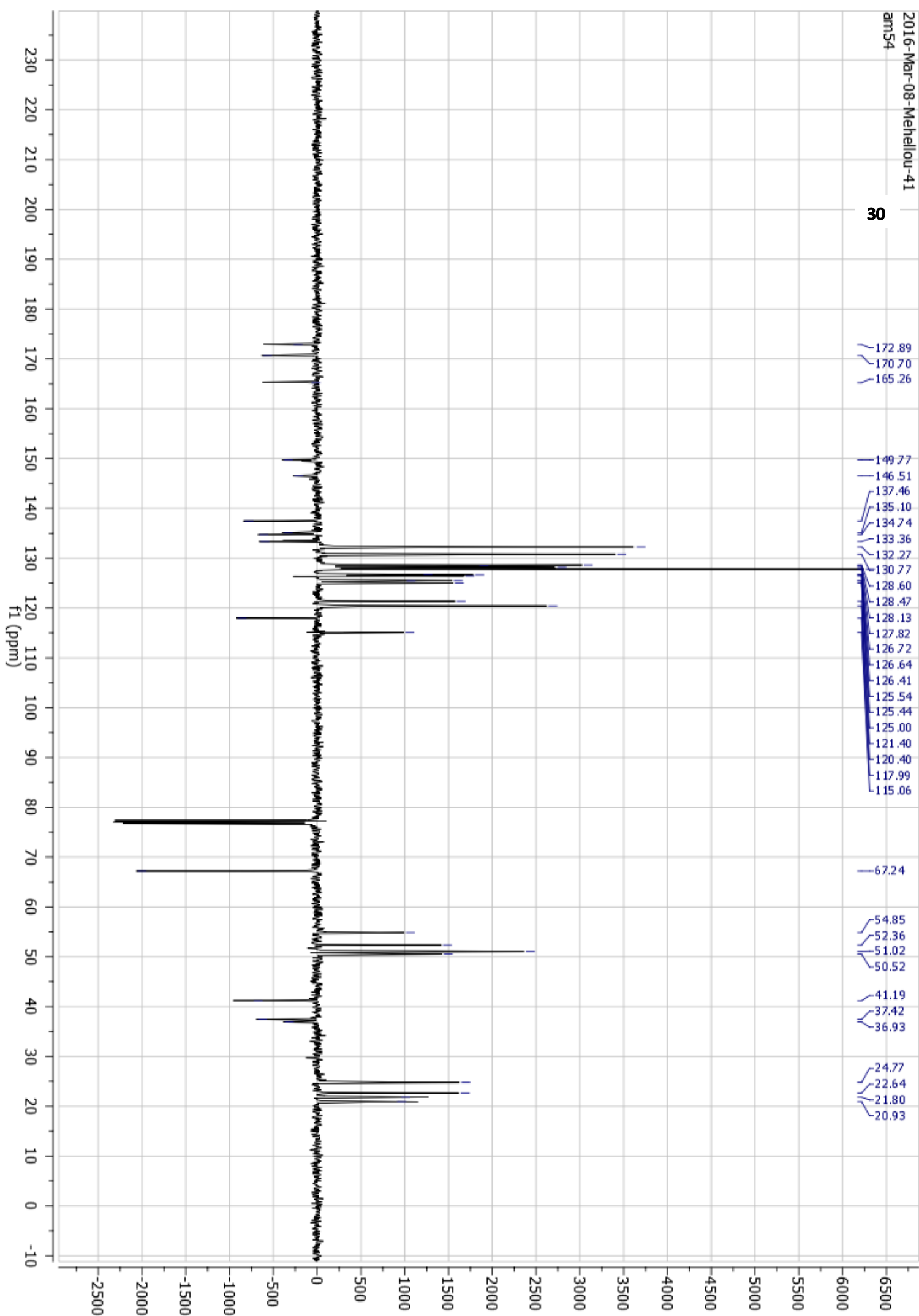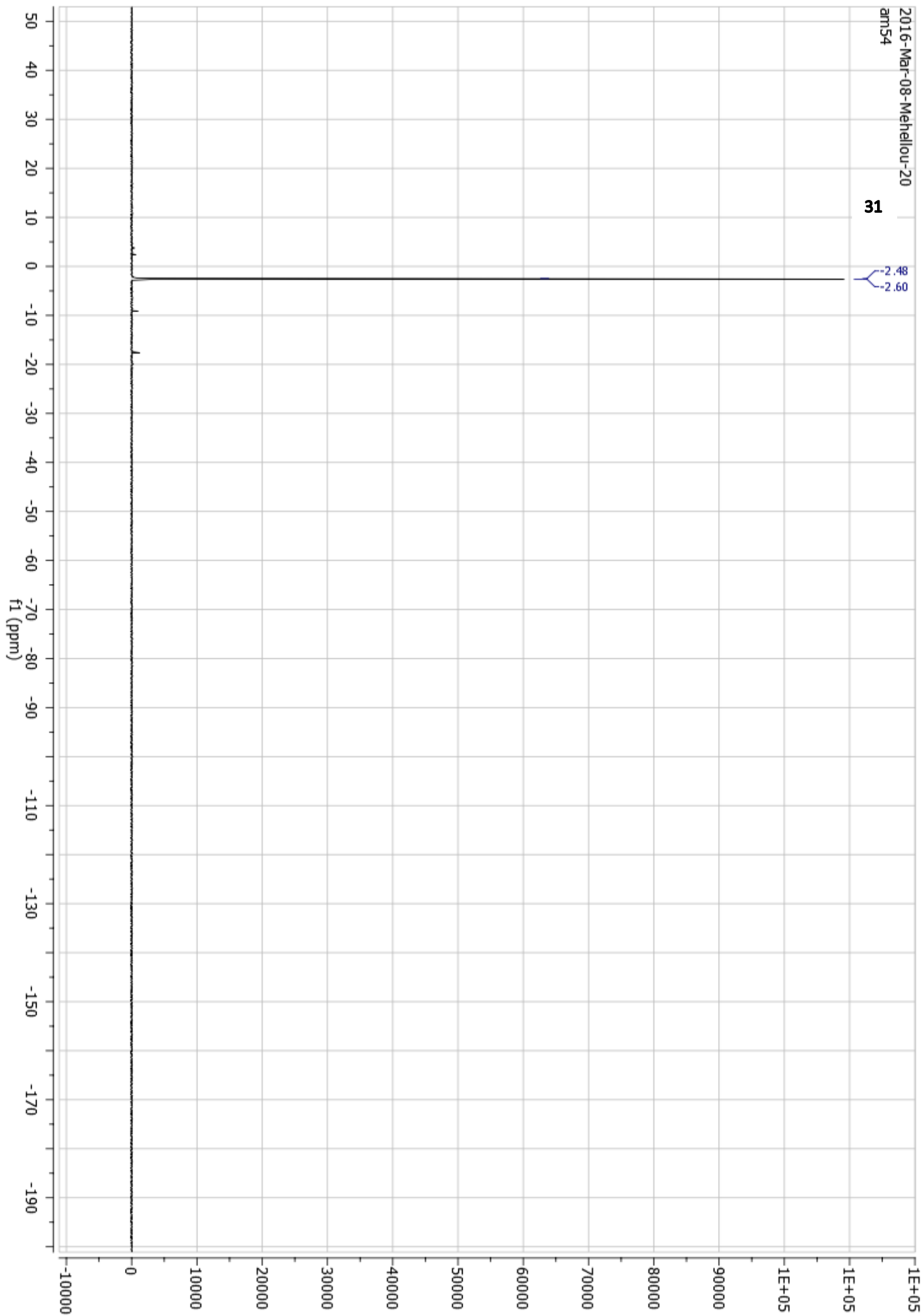
```
==================================================================
 Acq. Operator   : SYSTEM                    Seq. Line :   5
 Acq. Instrument : LC                         Location :   5
 Injection Date  : 6/30/2016 1:00:51 PM            Inj :   1
                                            Inj Volume : 10.000 µl
 Method          : C:\Chem32\1\Data\AllenB\AAB 30_06_16 AM 2016-06-30 09-28-02\AB Gradient
                   0to100 C=MeCN D=H2O.M (Sequence Method)
 Last changed    : 6/30/2016 9:28:02 AM by SYSTEM
 Additional Info : Peak(s) manually integrated
```
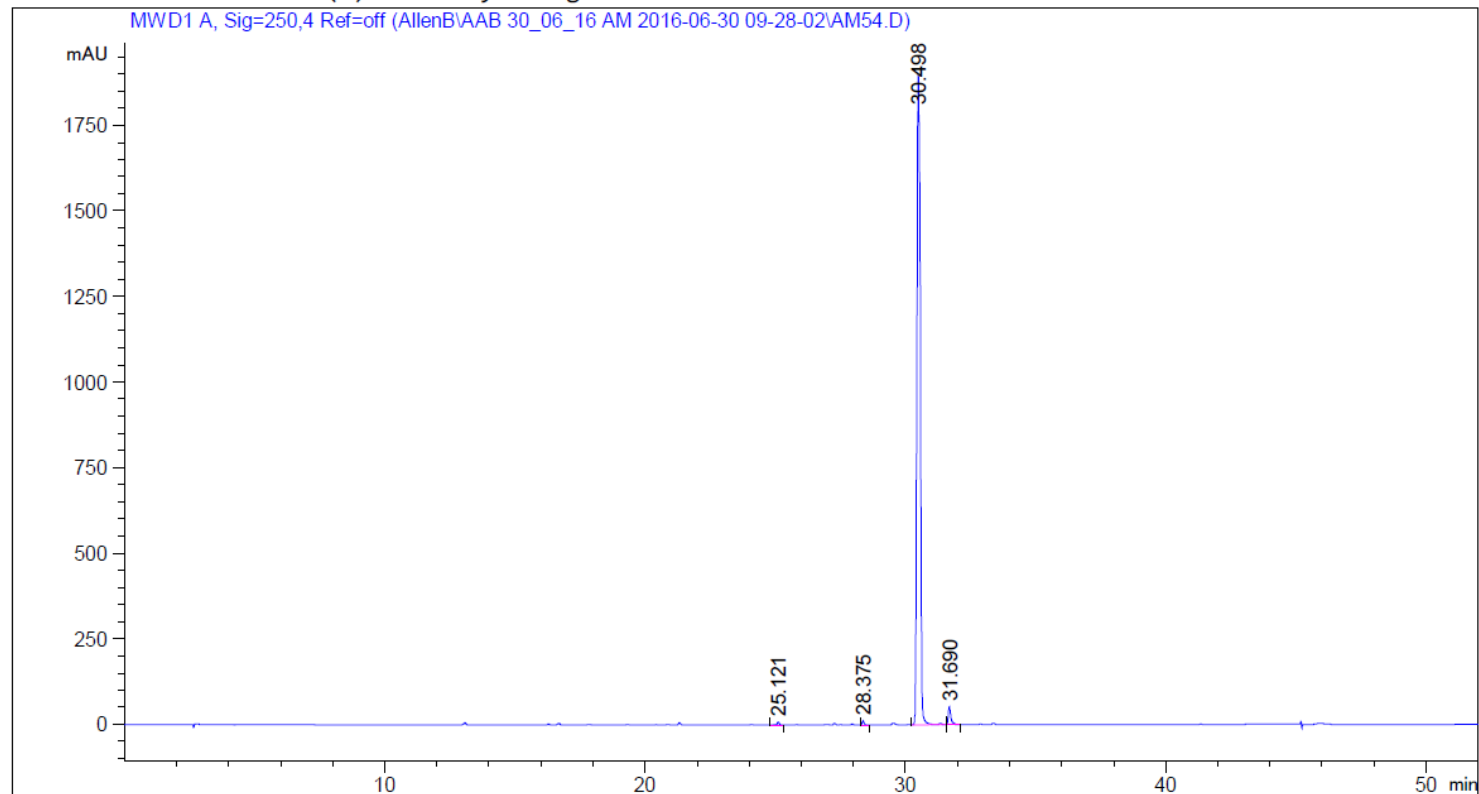
32

MWD1 A, Sig=250,4 Ref=off (AllenB\AAB 30_06_16 AM 2016-06-30 09-28-02\AM54.D)

```
==================================================================
                     Area Percent Report
==================================================================

 Sorted By             :      Signal
 Multiplier            :      1.0000
 Dilution              :      1.0000
 Use Multiplier & Dilution Factor with ISTDs


 Signal 1: MWD1 A, Sig=250,4 Ref=off

 Peak RetTime Type  Width     Area       Height     Area
  #   [min]        [min]   [mAU*s]      [mAU]       %
 ----|-------|----|-------|----------|----------|--------|
   1  25.121 VB R  0.0941   56.94046    8.96330    0.3202
   2  28.375 VB    0.0841   63.92260   11.63875    0.3594
   3  30.498 BV R  0.1281 1.72933e4  1897.75842   97.2416
   4  31.690 VB    0.1043  369.69379   52.41207    2.0788

 Totals :                 1.77838e4  1970.77254


==================================================================
```