Electronic Supplementary Material (ESI) for RSC Advances

Exploiting conformationally restricted *N*,*N'*-dimethyl-*N*,*N'*-diarylureas as biologically active C=C double bond analogues: synthesis and biological evaluation of combretastatin A-4 analogues

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Tubulin Polymerisation Inhibition (TPI).

Inhibition of tubulin polymerisation was evaluated by CytoDYNAMIX Screen™ 1 (Cytoskeleton Inc., Denver, CO) according to the manufacturer's instructions. Briefly, 100µl of 4 mg/ml HTS tubulin in G-PEM buffer plus 10% glycerol at 4°C was added to wells of a 96-well plate containing 10µM of individual compounds dissolved in dimethyl sulphoxide (DMSO) or blank controls containing the same concentration of DMSO (0.1%). Polymerisation was initiated by incubation at 37°C and absorption was read at 340 nm on a SpectraMax® Plus 384 Microplate Spectrophotometer (Molecular Devices, Inc. Sunnyvale, CA) every 60 sec for 60 min. Area under the curve (AUC) was used to calculate %TPI with AUC of the blank control set to 100% polymerization (maximal attainable polymerization). Data were analysed using GraphPad software v.5 (GraphPad Software, Inc., La Jolla, CA).

Cell Cultures and Treatment.

Two short-term glioblastoma multiforme (GBM) cell cultures (IN1472 and IN1760) derived in our laboratory from tumour biopsies as described by Lewandowicz *et al.*[1] and the established GBM cell line U251MG were used in this study. Cells were maintained in Hams F10 nutrient mix (Invitrogen Ltd., UK) with 10% foetal calf serum in a 37ºC non-CO_2 incubator. For growth inhibition and apoptosis assays, cells were seeded at a density of 10^3 cells/well into 96-well plates and incubated in growth media for 48 h prior to treatment. For the mitotic inhibition assay, cells were seeded into 8-well chamber slides at a density of 2000 cells/well and incubated in growth media for 48 h prior

to treatment. CA4P and the 5 analogues were dissolved in DMSO and all assays were carried out using 10 μM of each compound.

Mitotic Inhibition Assay.

Cells were incubated in growth media containing test compounds for 24 hours prior to fixation in 3 changes of methanol : acetic acid (3:1) supplemented with 4mM MG+ and 1mM Ca+ ions, as previously described.[2] Cells were differentially stained with Brilliant blue R and Safranin O which stain the mitotic spindle and chromosomes, respectively. The mitotic index was determined on 1000 cells using standard bright-field microscopy and compared to DMSO-treated controls for each cell culture using Chi-square and tests with Yates correction at the 95% confidence limits.

Growth Inhibition Assay.

The anti-proliferative effects of the compounds were determined by 3-(4,5-dimethylthiazol-2-yl)-2,5—diphenyltetrazolium bromide (MTT) assay as previously described.[1] Briefly, cells were incubated for 72 h at 37ºC in the presence of 10μM of each compound with renewal of compounds at 24 h intervals. Each compound was tested in quadruplicate. Cells were then allowed a 24-h recovery period at 37 °C in compound-free media before media was aspirated and 100 μl of MTT solution (1 mg/ml in Ham's F10 medium without FCS) was added to each well. Four hours later, formazan crystals were dissolved in DMSO (100 μl/well) and the plates were agitated for 2-3 min before the optical density at 570 nm was determined on a plate reader (Fluoroskan Ascent, Thermo Labsystems, Cheshire, UK). Results were expressed as percentage of growth inhibition compared to DMSO-treated controls for each compound (student's t-test at 95% confidence limits).

Apoptosis Assay.

Cells were treated in triplicate with each compound for 24 h before determining the proportion of apoptotic cells using cell death detection ELISA^{Plus} (Roche Diagnostic Limited, West Sussex, UK). Briefly, 20μl of cell lysate was incubated with 80μL of anti-histone-biotin and anti-DNA-peroxidase for 2 hours at room temperature. The unbound antibodies were then washed off and the peroxidase was developed using ABTS as substrate before determining optical density at 405nm on a plate reader (Fluoroskan Ascent, Thermo Labsystems, Cheshire, UK). Results were expressed as an apoptotic index (AI) where AI = OD drug treated cells/OD DMSO control cells. Data was analysed using student's t-test at 95% confidence limits.

General Synthetic Procedures.

Commercially available reagents were used as received without purification. Analytical thin layer chromatography (TLC) was performed with plastic-backed TLC plates coated with silica G/UV_{254}, in a variety of solvents. The plates were visualised by UV light (254 nm). Flash column chromatography was conducted with Davisil silica 60Å (40-63 μm) under bellows pressure. Low resolution mass spectra were recorded on a Thermo Finnigan LCQ Advantage MAX using chemical ionisation (CI). Melting points were measured on a Stuart SMP10 melting point apparatus and are uncorrected. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker Avance DPX 250 (250 MHz) or a Bruker 400 (400 MHz) spectrometer. All chemical shifts (δ) are quoted in parts per million (ppm) relative to a calibration reference of the residual protic solvent; C*H*Cl_3 (δ_H 7.26, s) was used as the internal standard in ^{1}H NMR spectra, and ^{13}C NMR shifts were referenced using *C*DCl_3 (δ_C 77.0, t) with broad band decoupling and the *J* values are measured in Hertz.

General synthesis of *N,N'*-dimethyl- *N,N'*-diarylureas 11-14.

The aniline **5**-**8** (1 equiv) and isocyanate **9** or **10** (1 equiv) were dissolved in dichloromethane (0.1M) and stirred at room temperature for 16 hours. Upon completion of the reaction (TLC) the solvent was evaporated *in vacuo* to yield the diarylurea as a semi-crude solid. The urea was suspended in tetrahydrofuran (0.1M) and sodium hydride (60% in mineral oil, 1.5 equiv per NH) was added and the reaction stirred at room temperature for 5 minutes. Methyl iodide (1.5 equiv per NH) was added and the reaction stirred at room temperature until determined complete (TLC). The solvent was evaporated *in vacuo* and the residue dissolved in ethyl acetate. The product was washed with 1M HCl, brine, dried ($MgSO_4$) and the solvent evaporated *in vacuo*. The crude product was purified by flash column chromatography or trituration.

N,N'-Dimethyl-N,N'-diphenylurea, 11.

Purified by trituration with 10% DCM in petroleum ether to yield the title compound (70%, 2 steps) as a pale yellow solid; m.p. (10% DCM:petroleum ether) 121-123 °C; Found: C 74.77; H, 6.86; N, 11.63. $C_{15}H_{16}N_2O$ requires C 74.97; H, 6.71; N, 11.66%; ν_{max}/cm^{-1} 1645, 1584, 1495, 1428, 1358; δ_H (250 MHz; $CDCl_3$) 7.06-6.99 (4H, m, Ar), 6.94-6.88 (2H, m, Ar), 6.80-6.75 (4H, m, Ar), 3.18 (6H, s, 2 × -NMe); δ_C (62.5 MHz; $CDCl_3$) 161.2, 145.5 (C), 128.5, 125.6, 124.7 (CH), 39.2 (CH_3); *m/z* (CI) 241 ($[M+H]^+$, 100%).

N-(4-Methoxyphenyl)-N,N'-dimethyl-N'-phenylurea, 12.

Purified by flash chromatography with DCM to yield the title compound (62%, 2 steps) as an off-white solid; m.p. (DCM) 92-93 °C; Found: C 70.93; H, 6.90; N, 10.34. $C_{16}H_{18}N_2O_2$ requires C 71.09; H, 6.71; N, 10.36%; ν_{max}/cm^{-1} 1646, 1594, 1507, 1453, 1431, 1352; δ_H (250 MHz; $CDCl_3$) 7.05-7.01 (2H, m, Ar), 6.96-6.89 (1H, m, Ar), 6.79-6.74 (2H, m, Ar), 6.69-6.63 (2H, m, Ar), 6.58-6.52 (2H, m, Ar), 3.69 (3H, s, -OMe), 3.15 (3H, s, -NMe), 3.13 (3H, s, -NMe); δ_C (62.5 MHz; $CDCl_3$) 161.5, 156.8, 145.8, 138.6 (C), 128.6, 127.2, 125.7, 124.8, 113.8 (CH), 55.3, 39.7, 39.4 (CH_3); *m/z* (CI) 271 ($[M+H]^+$, 100%).

N,N'-Dimethyl-N-phenyl-N'-(3,4,5-trimethoxyphenyl)urea, 13.

Purified by trituration with 10% DCM in petroleum ether to yield the title compound (74%, 2 steps) as a pale yellow solid; m.p. (10% DCM:petroleum ether) 100-101 °C; Found: C 65.42; H, 6.90; N, 8.40. $C_{18}H_{22}N_2O_4$ requires C 65.44; H, 6.71; N, 8.48%; ν_{max}/cm^{-1} 1649, 1588, 1503, 1454, 1348; δ_H (250 MHz; $CDCl_3$) 7.10-7.03 (2H, m, Ar), 7.00-6.94 (1H, m, Ar), 6.79-6.76 (2H, m, Ar), 5.93 (2H, s, Ar), 3.72 (3H, s, -OMe), 3.65 (6H, s, 2 × -OMe), 3.18 (3H, s, -NMe), 3.15 (3H, s, -NMe); δ_C (62.5 MHz; $CDCl_3$) 161.3, 152.9, 145.6, 141.4, 135.5 (C), 128.6, 126.4, 125.0, 104.1 (CH), 60.7, 55.9, 39.6, 39.5 (CH_3); *m/z* (CI) 331 ($[M+H]^+$, 100%).

N-(4-Methoxyphenyl)-N,N'-dimethyl-N'-(3,4,5-trimethoxyphenyl)urea, 14.

Purified by flash chromatography with 10% petroleum ether in ethyl acetate to yield the title compound (54%, 2 steps) as a pale yellow solid; m.p. (10% petroleum ether:ethyl acetate) 91-93 °C; Found: C 63.16; H, 6.92; N, 7.75. $C_{19}H_{24}N_2O_5$ requires C 63.32; H, 6.71; N, 7.77%; ν_{max}/cm^{-1} 2940, 1650, 1592, 1505; δ_H (250 MHz; $CDCl_3$) 6.65 (2H, d, J = 9.0, Ar), 6.58 (2H, d, J = 9.0, Ar), 5.91 (2H, s, Ar), 3.73 (3H, s, -OMe), 3.68 (3H, s, -OMe), 3.65 (6H, s, 2 × -OMe), 3.13 (3H, s, -NMe), 3.11 (3H, s, -NMe); δ_C (62.5 MHz; $CDCl_3$) 161.6, 157.0, 152.9, 141.7, 138.6, 135.6 (C), 127.8, 113.8, 104.1 (CH), 60.7, 55.9, 55.3, 39.81, 39.79 (CH_3); *m/z* (CI) 361 ($[M+H]^+$, 100%).

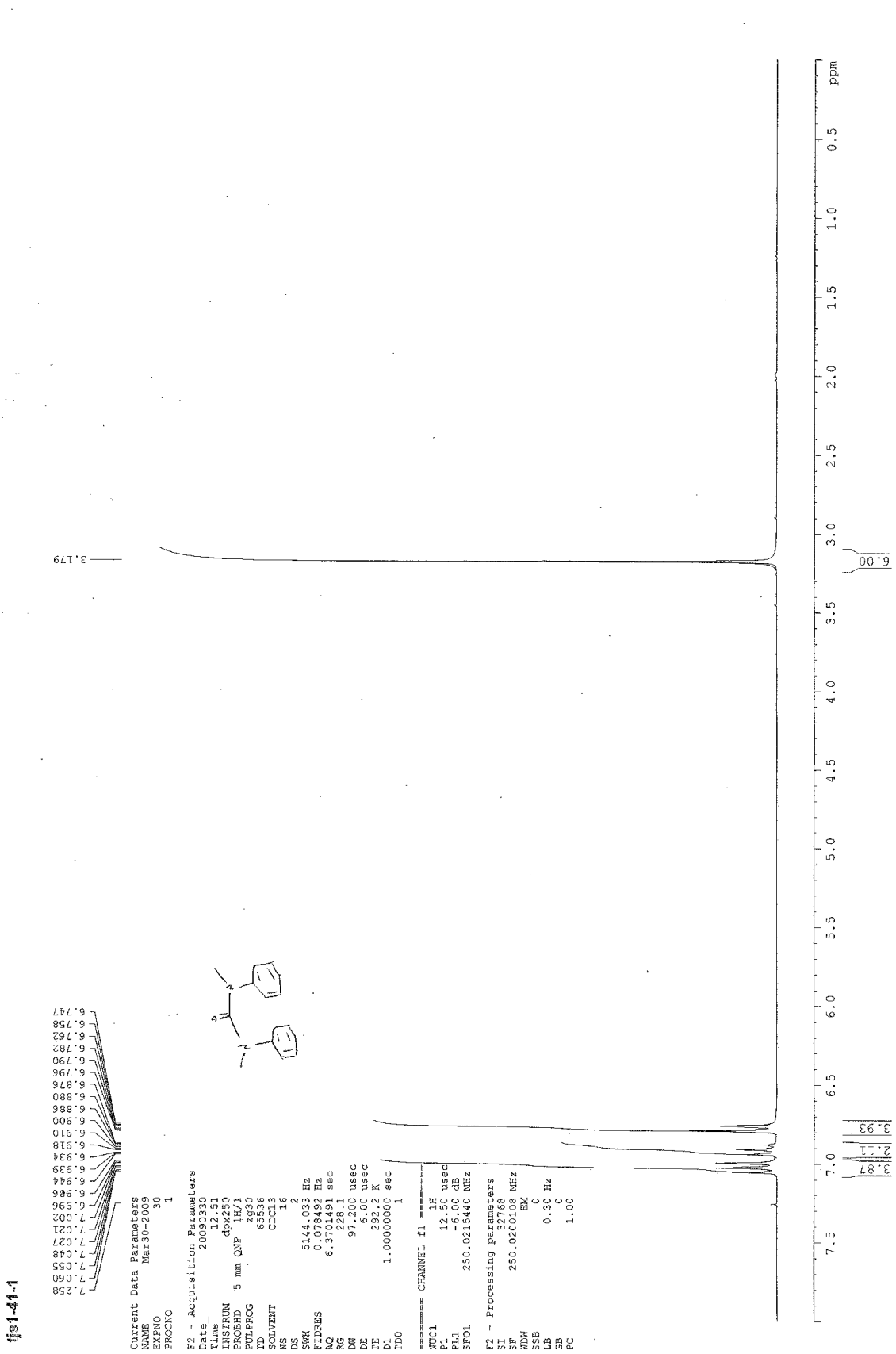
N-(4-Methoxyphenyl)-N-methyl-N'-(3,4,5-trimethoxyphenyl)urea, 15.

4-Methoxy-*N*-methylaniline (328 mg, 2.39 mmol) and 3,4,5-trimethoxyphenyl isocyanate (500 mg, 2.39 mmol) were dissolved in dichloromethane (24 ml) and stirred at room temperature for 16 hours. Upon completion of the reaction (TLC) the reaction mixture was washed with 1M HCl (2 × 20 ml), water (20 ml), brine (20 ml), dried ($MgSO_4$) and the solvent evaporated *in vacuo*. The crude product was purified by flash column chromatography (SiO_2, 50% ethyl acetate in petroleum ether) to yield the title compound (674 mg, 81%) as a colourless solid; m.p. (50% petroleum ether:ethyl acetate) 140-142 °C; Found: C 62.35; H, 6.37; N, 8.03. $C_{18}H_{22}N_2O_5$ requires C 62.42; H, 6.40; N,

8.09%; ν_{max}/cm^{-1} 3353, 2933, 2830, 1658, 1603, 1528, 1506; δ_H (250 MHz; $CDCl_3$) 7.26 (2H, d, *J* = 8.9, Ar), 7.00 (2H, d, *J* = 8.9, Ar), 6.59 (2H, s, Ar), 6.13 (1H, br. s, NH), 3.86 (3H, s, -OMe), 3.81 (6H, s, 2 ×-OMe), 3.77 (3H, s, -OMe), 3.30 (3H, s, -NMe); δ_C (62.5 MHz; $CDCl_3$) 159.0, 154.7, 153.1, 135.08, 135.07, 133.4 (C), 128.8, 115.4, 96.6 (CH), 60.9, 56.0, 55.5, 37.4 (CH_3); *m/z* (CI) 347 ($[M+H]^+$, 100%).

References

1. G. M. Lewandowicz, B. Harding, W. Harkness, R. Hayward, D. G. T. Thomas and J. L. Darling, *Eur. J. Cancer*, 2000, **36**, 1955-1964.
2. T. J. Warr, E. M. Parry and J. M. Parry, *Mutation Research*, 1993, **287**, 29-46.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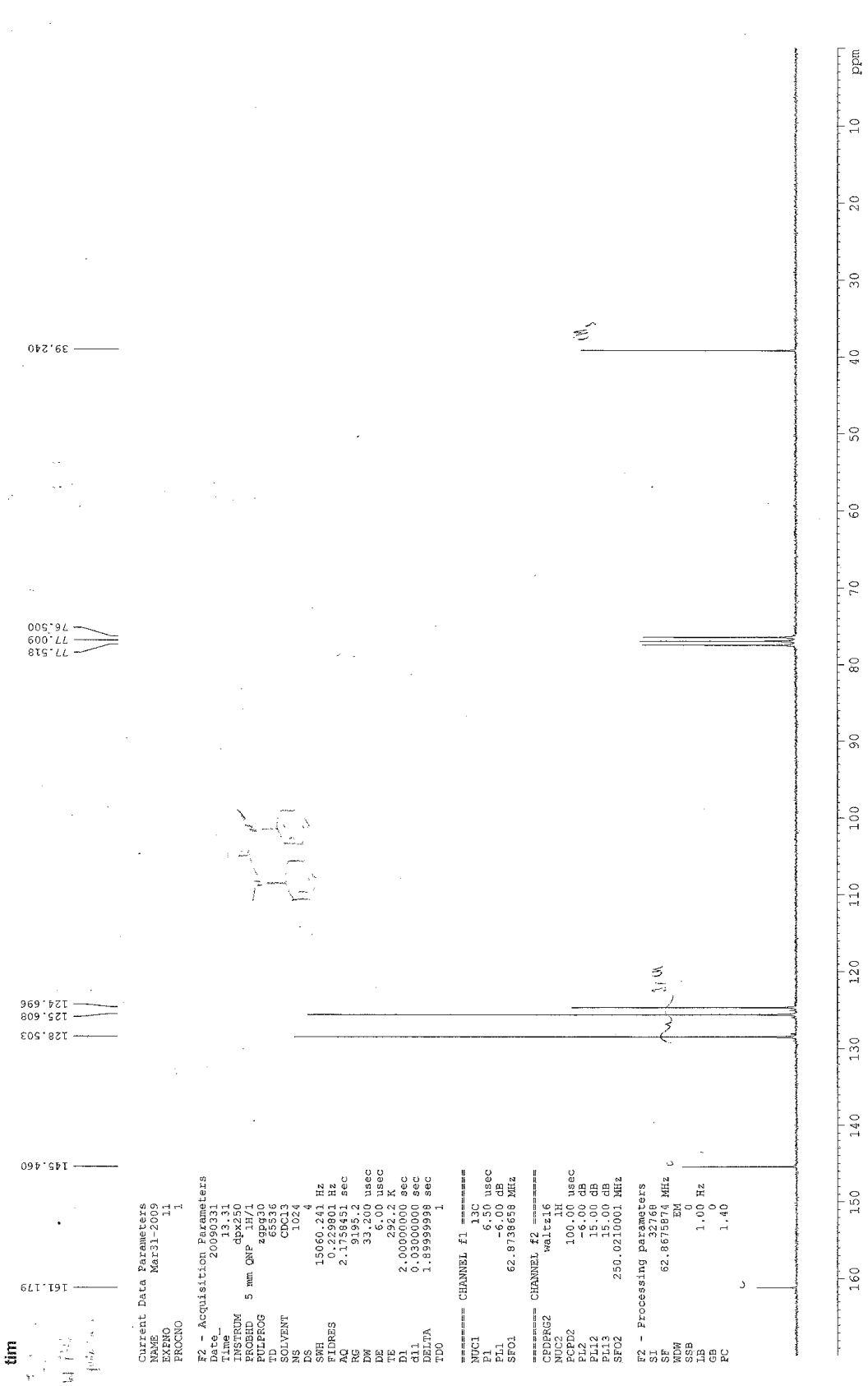
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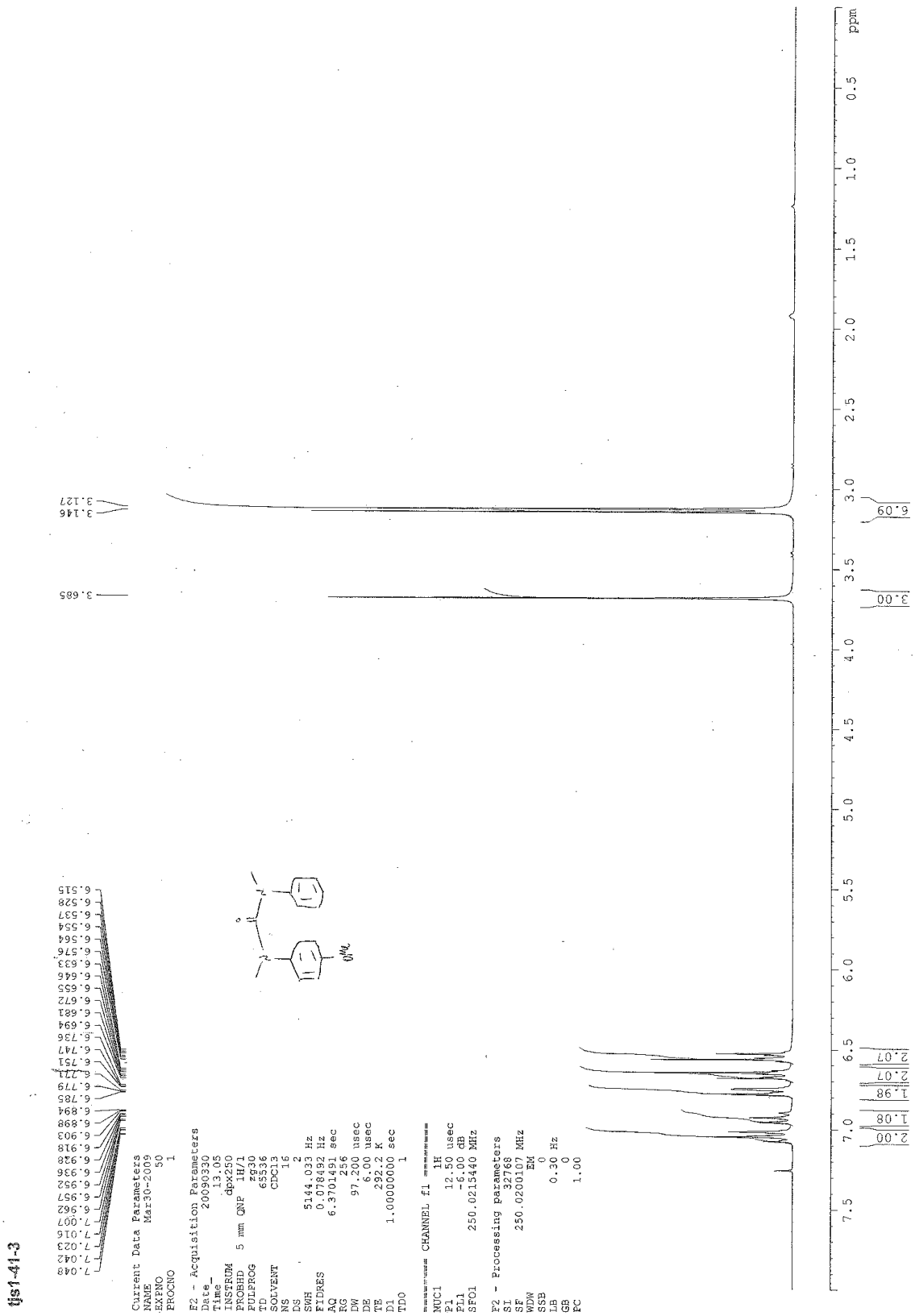
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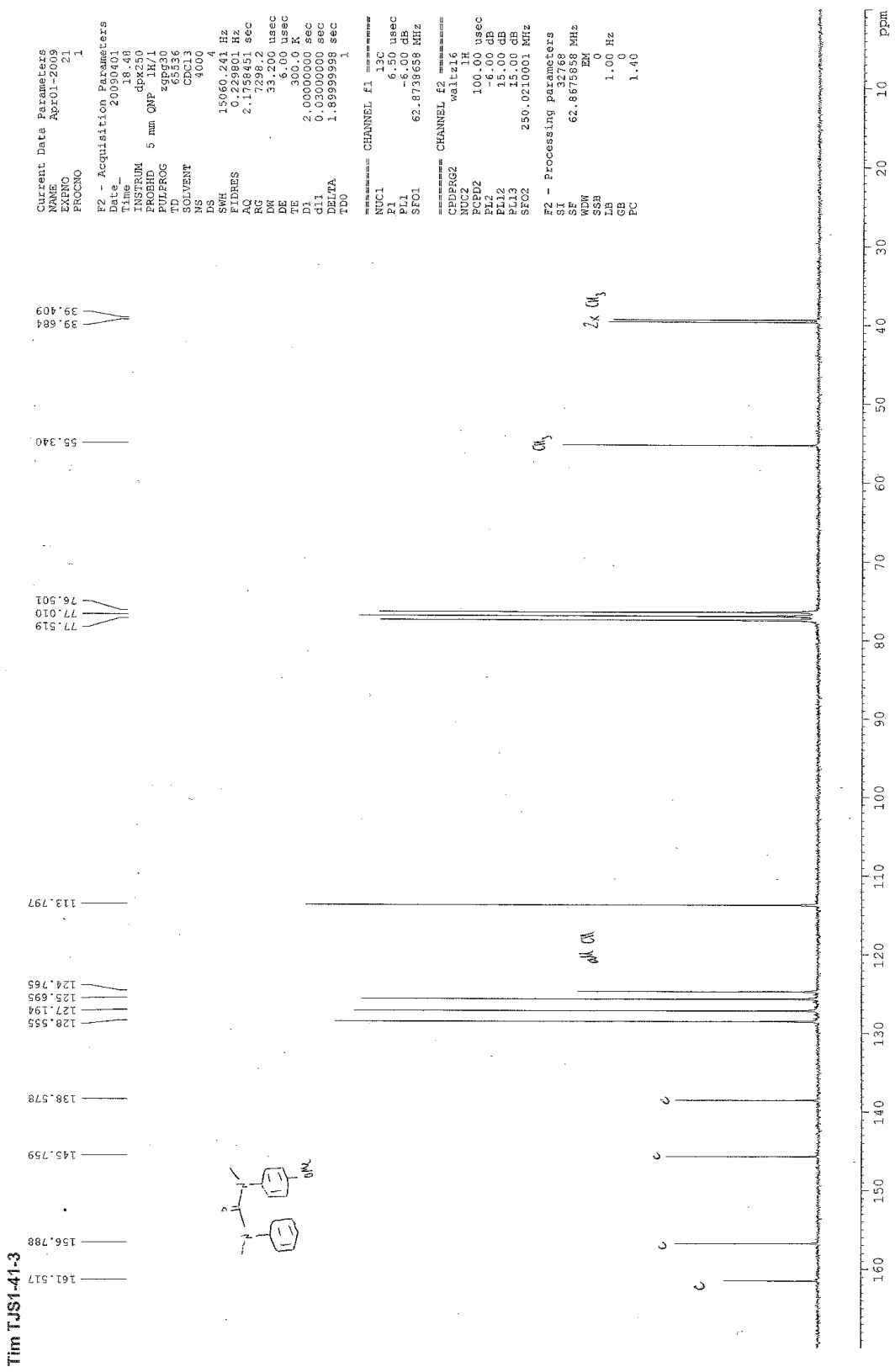

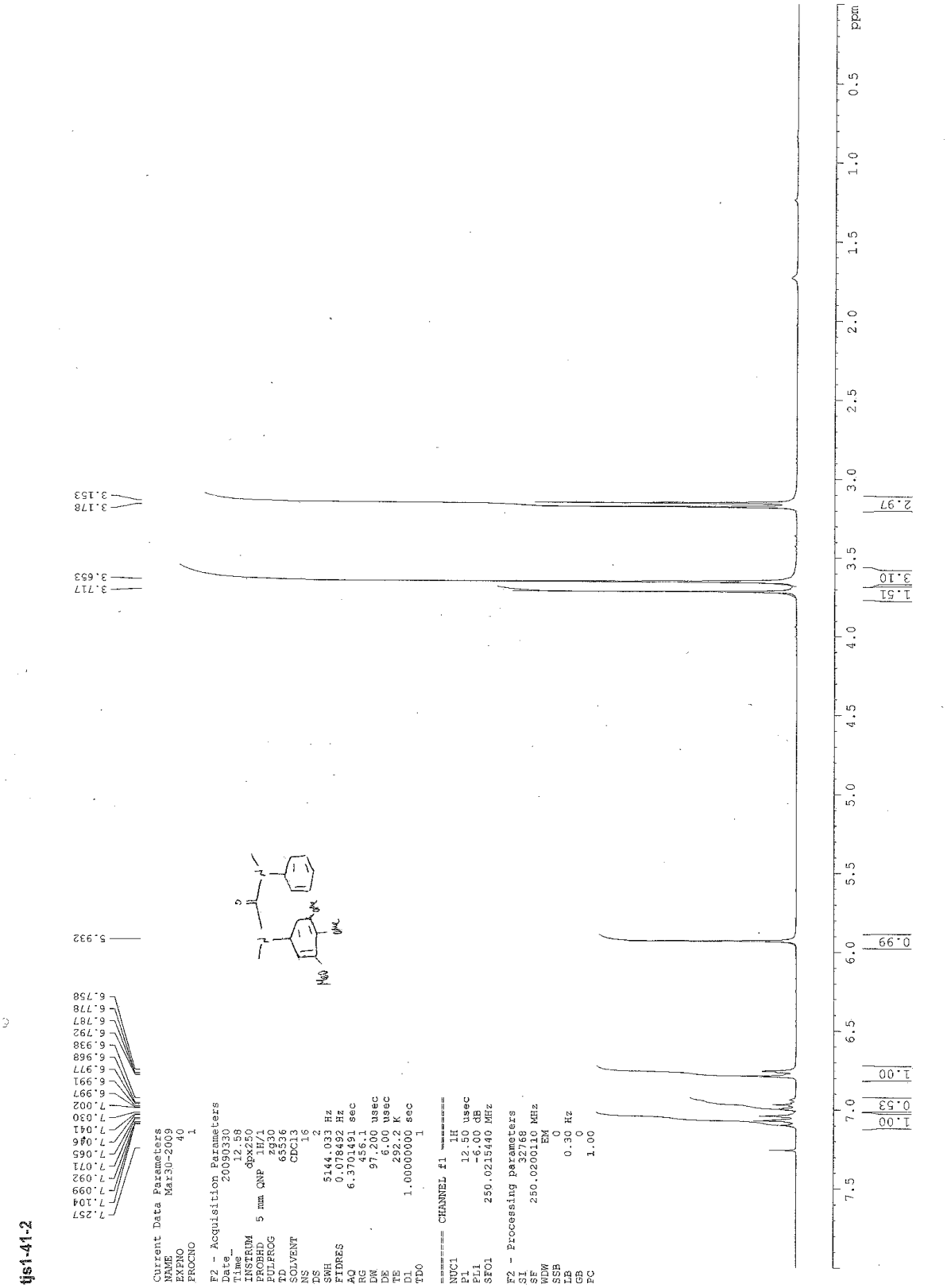
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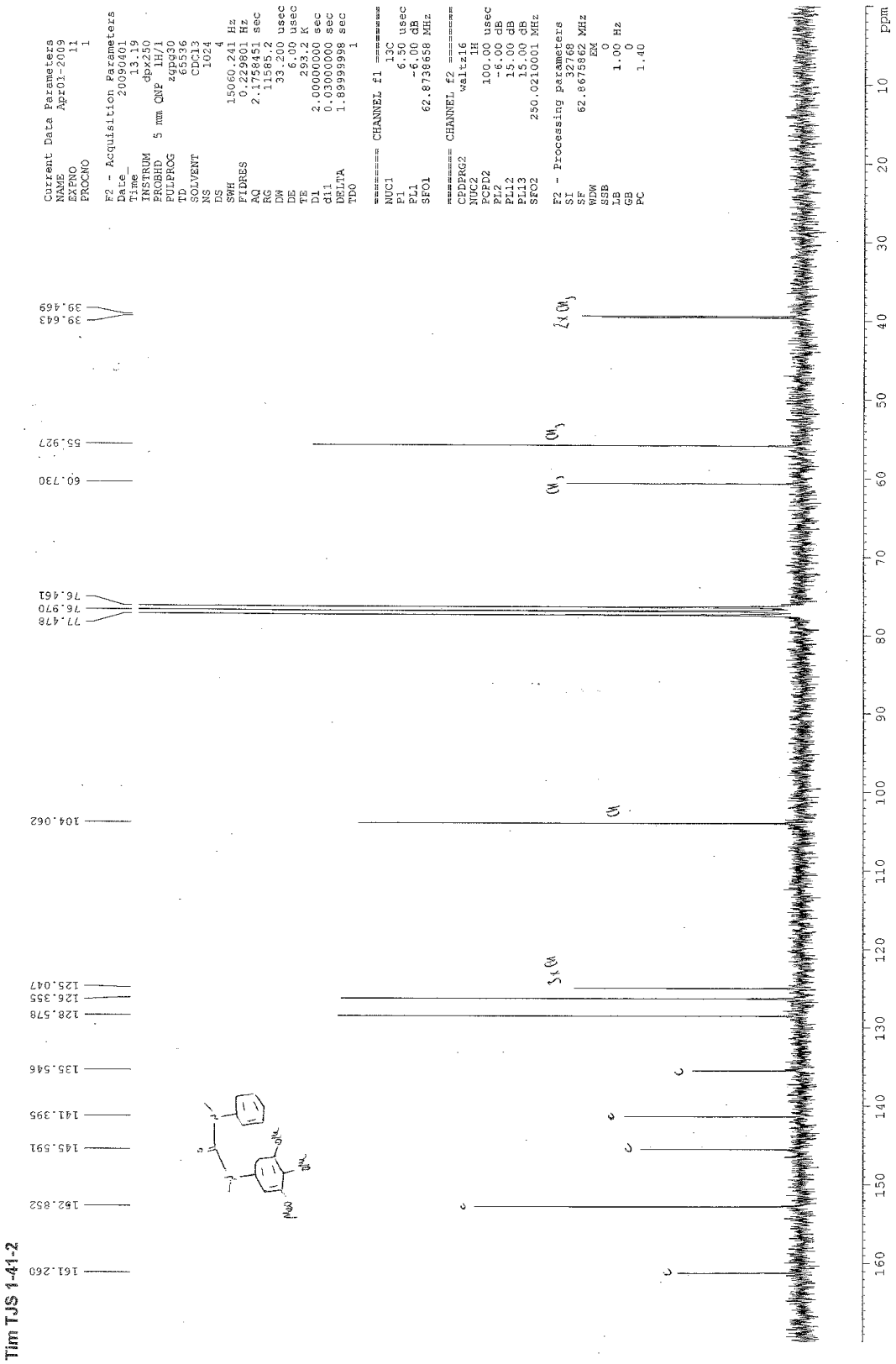
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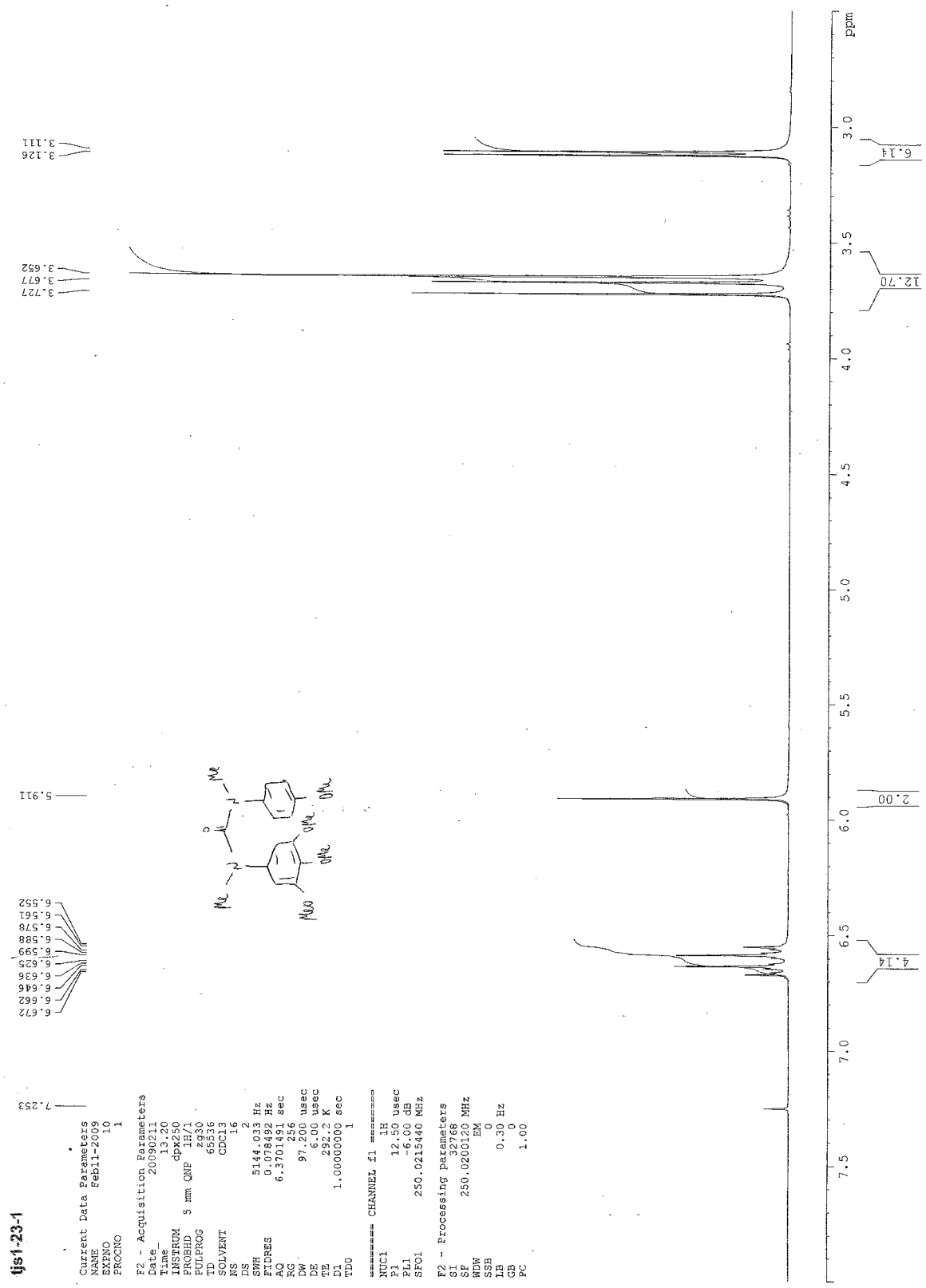
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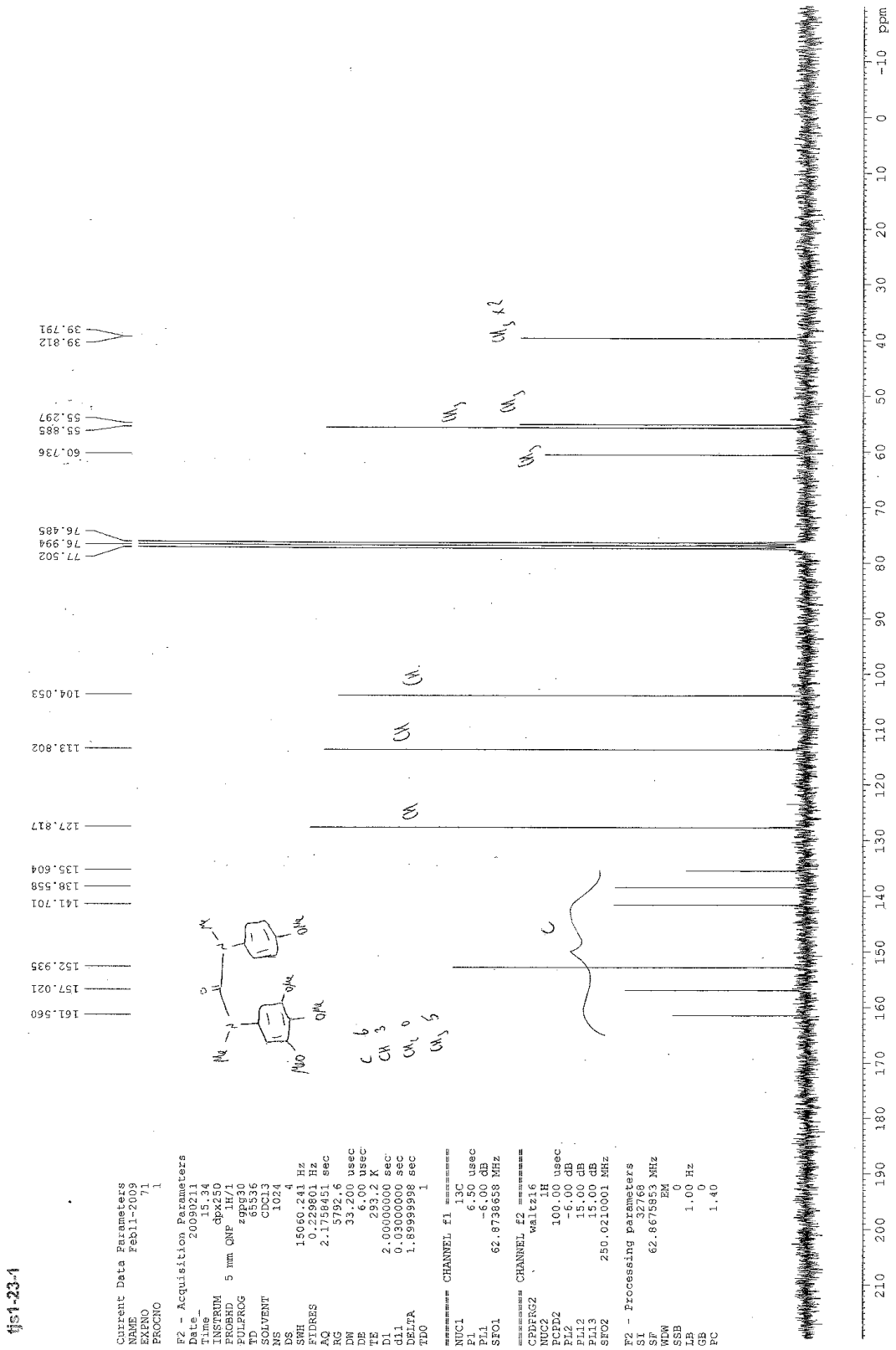

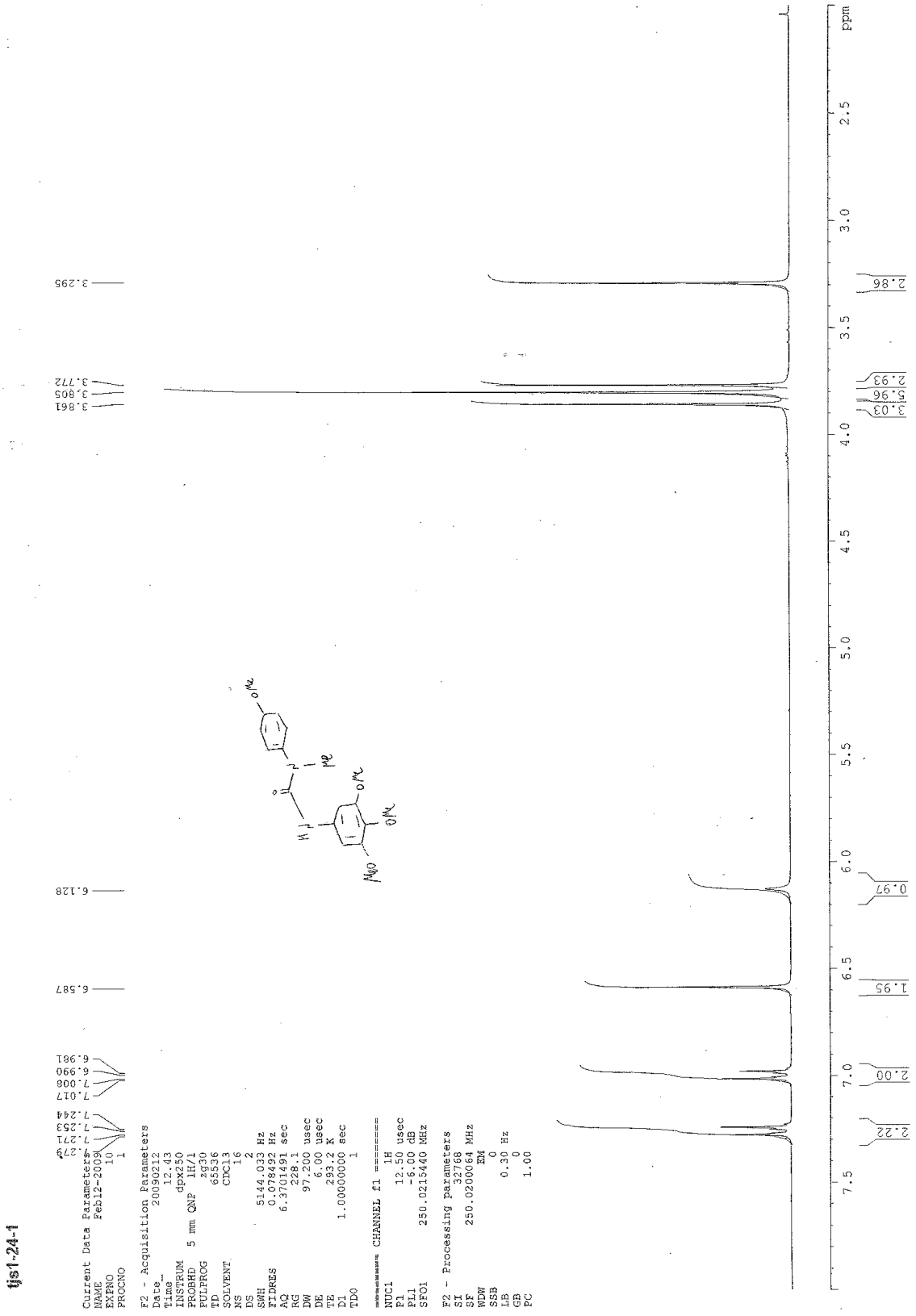
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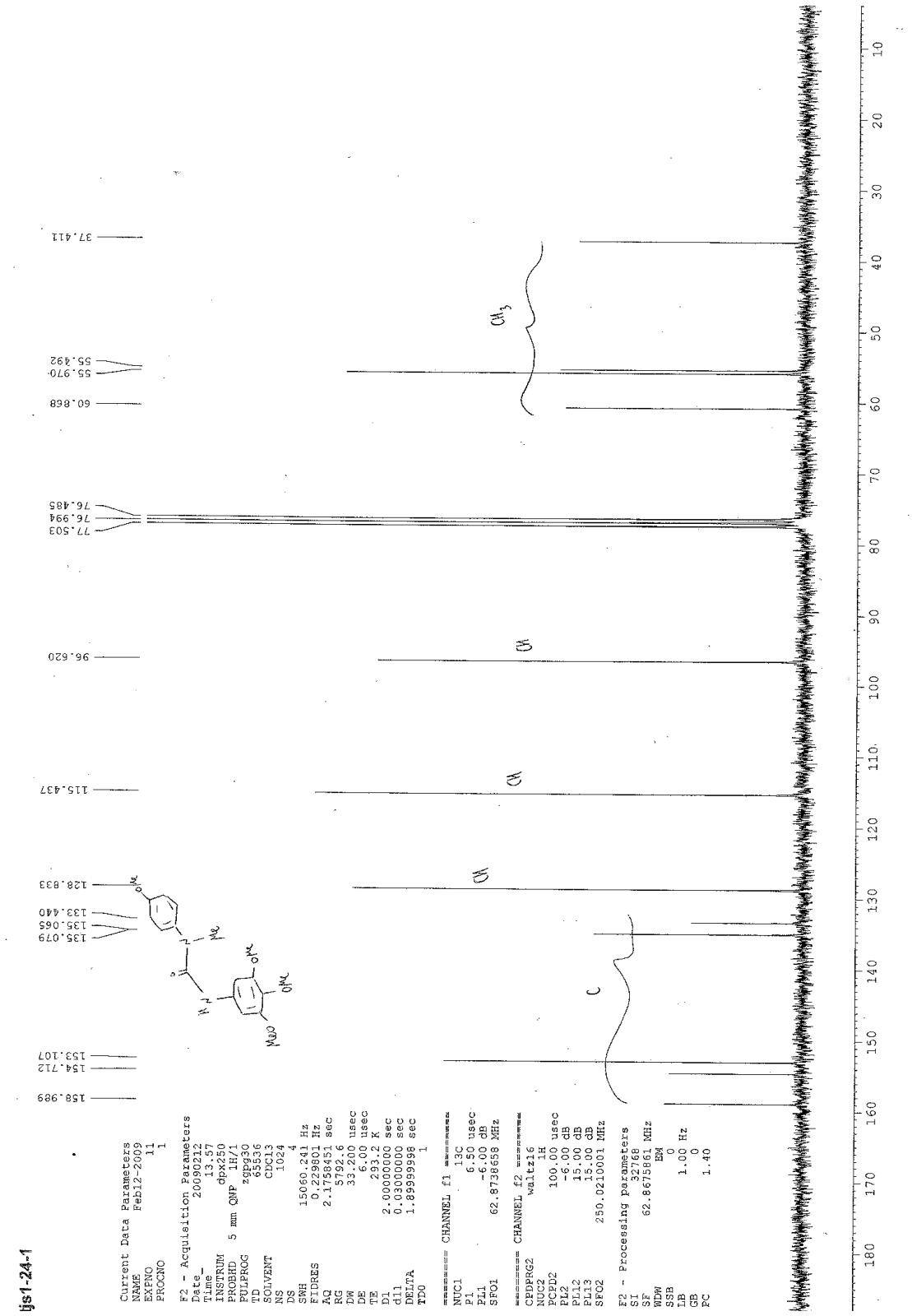
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