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for

Rapid Identification of Potent Nonpeptidic Serine Protease Inhibitors

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Materials. Unless otherwise noted, chemicals were obtained from commercial suppliers and used without further purification. Wang polystyrene resin was purchased from Novabiochem (San Diego, CA). Anhydrous, low amine content *N, N'*-dimethylformamide (DMF) was purchased from EM Science (Cincinnati, OH). Chymotrypsin (bovine pancreatic, TLCK-treated) and trypsin (bovine pancreatic, TPCK-treated) were purchased from Sigma (St. Louis, MO). Cathepsin G, human neutrophil elastase (HNE), *Z*-Phe-Arg-AMC, Suc-Ala-Ala-Pro-Phe-AMC, and MeOSuc-Ala-Ala-Pro-Val-AMC were purchased from Calbiochem (San Diego, CA). Dichloromethane, pyridine, acetonitrile, and diisopropylethylamine were distilled from calcium hydride under N_2. THF was distilled from sodium/benzophenone ketyl under N_2. Fmoc-protected 7-amino-4-methylcoumarin-3-acetic acid (Fmoc-AMCA-OH) was prepared as reported.[1] Fmoc quantitation was performed according to literature procedure.[2] The enzyme solution concentrations were based on total weighed protein mass. Unless otherwise noted, all spectra were obtained in [D_6]-DMSO. NMR chemical shifts are reported in ppm downfield relative to the internal solvent peak, and *J* values are in Hz.

Initial screening of diverse nonpeptidyl AMCA substrates. Substrate stock solutions were prepared in DMSO. The substrates were diluted in assay buffer (50 mM Tris pH 8.1, 1 mM EDTA, and 0.01% tween) to a final concentration of 250 μM. The concentration of DMSO in the assays was less than 5%. The final chymotrypsin concentration was 250 nM. Hydrolysis of AMCA substrates was monitored fluorometrically with an excitation wavelength of 370 nm and emission wavelength of 455 nm on a Fluoromax-2 spectrofluorimeter (Molecular Devices, Sunnyvale, CA). All individual rates were measured in duplicate at 37 ºC, and the rate of non-enzymatic background hydrolysis was subtracted.

Fmoc-AMCA-Wang resin 2. oWang polystyrene resin (**1**, 3.0 g, 3.0 mmol) was added to a fritted syringe, swollen with CH_2Cl_2, and washed with anhydrous DMF (40 mL). A solution of Fmoc-AMCA-OH (3.14g, 6.90 mmol) in DMF (23 mL) was added to the resin, followed by pyridine (0.93 mL, 11 mmol) and 2,6-dichlorobenzyl chloride (0.99 mL, 6.9 mmol). The resin was agitated for 6 days and then was thoroughly washed with DMF (3 x 40 mL), THF (3 x 40 mL), MeOH (3 x 40 mL), THF (3 x 40 mL), and CH_2Cl_2 (3 x 40 mL). Fmoc quantitation of the resulting resin **2** indicated that the loading of AMCA was 79% (0.54 mmol/g).

Acrylamide-AMCA-Wang resin 3. To prepare resin **3**, Fmoc-AMCA-Wang polystyrene resin (**2**, 2.5 g, 1.4 mmol) was added to a fritted syringe and swollen with DMF. The Fmoc group was removed by treatment with 20% piperidine in DMF (30 mL) for 30 min. The deprotected resin was filtered and thoroughly washed with DMF (3 x 40 mL), THF (3 x 40 mL), MeOH (3 x 40 mL), THF (3 x 40 mL), and CH_2Cl_2 (3 x 40 mL).

To a 100 mL round-bottom flask under N_2 fitted with a stir bar was added the deprotected resin and CH_2Cl_2 (20 mL). The resin was stirred gently, and *i*-Pr_2EtN (1.6 mL, 9.1 mmol) was added dropwise. The solution was cooled to 0 ºC, and acryloyl chloride (0.55 mL, 6.8 mmol) was added dropwise. The resin was agitated for 24 h and was thoroughly washed with DMF (3 x 40 mL), THF (3 x 40 mL), MeOH (3 x 40 mL), THF (3 x 40 mL), and CH_2Cl_2 (3 x 40 mL) to provide resin **3**.

General procedure for the preparation of oximes. To individual 20 mL vials fitted with stir bars were added aldehyde (3.30 mmol), EtOH (6 mL), and hydroxylamine hydrochloride (274 mg, 4.00 mmol). The reaction mixtures were stirred overnight, and the solution mixtures were concentrated. A 10% citric acid solution (3 mL) was added to each vial, and the reaction mixtures were extracted with CH_2Cl_2 (3 x 2 mL). The combined organic layers were concentrated to provide the corresponding oximes, which were used without further purification.

Aldehyde	Amount used (x mg, 3.30 mmol)
3-tolualdehyde	396
cyclohexanecarboxaldehyde	370
2-fluorobenzaldehyde	410
4-tolualdehyde	396
4-trifluoromethylbenzaldehyde	575
4-anisaldehyde	449
3-fluorobenzaldehyde	410
transcinnamylaldehyde	436
4-biphenylcarboxaldehyde	601

1-napthaldehyde	515
2-tolualdehyde	396
3-phenylproprionaldehyde	443
4-fluorobenzaldehyde	410
2-napthaldehyde	515
pentafluorobenzaldehyde 2-chloro, 6-fluorobenzaldehyde	647 523
2, 6-difluorobenzaldehyde	469
2, 4-difluorobenzaldehyde	469
2, 5-difluorobenzaldehyde	469
2-fluoro,3-trifluoromethylbenzaldehyde	624
5-bromo, 2-fluorobenzaldehyde	671
2-fluoro, 6-trifluoromethylbenzaldehyde	624
2-anisaldehyde	449

General procedure for the preparation of 3-substituted isoxazoline-AMCA substrates (5a-5x). The synthesis of 3-substitutes isoxazoline substrates began with the preparation of the corresponding hydroximoyl chlorides. The hydroximoyl chlorides were prepared by adding added oxime (*vida supra*, 0.81 mmol), CH_2Cl_2 (1.1 mL) and THF (0.5 mL) to individual 3 mL fritted syringes. *N*-chlorosuccinimide (108 mg, 0.81 mmol) and pyridine (9 μL. 0.1 mmol) were added to each fritted syringe, and the solutions were agitated. After 2 h, resin **3** (130 mg, 0.70 mmol) and *i*-Pr_2EtN (0.14 mL, 0.81 mmol) were added to each fritted syringe. The reaction mixtures were agitated for 2 h, filtered, and thoroughly washed with DMF (3 x 2 mL), THF (3 x 2 mL), MeOH (3 x 2 mL), THF (3 x 2 mL), and CH_2Cl_2 (3 x 2 mL) to provide support-bound isoxazoline substrates **4a**-**4x**. The substrates were cleaved from support by incubation with 95:2.5:2.5 TFA/*i*-Pr_2SiH/H_2O (1.5 mL) for 1 h. The resulting substrates **5a**-**5x** were purified using preparatory C18 reversed-phase HPLC.

3-Phenyl-isoxazoline AMCA substrate 5a (CSVI132D). The substrate was synthesized according to the general procedure for the preparation of 3-substituted isoxazoline-AMCA substrates (*vida supra*) and purified using preparatory C18 reversed-phase HPLC (CH_3CN/H_2O-0.1% TFA, 5-95% for 50 min, 8 mL/min, 220/254/280 nm detection for 70 min, t_R: 34.4 min). The substrate was ≥99% pure as determined by HPLC-MS analysis (CH_3CN/H_2O-0.1% TFA, 5-95% for 14 min, 0.4 mL/min, 220/254/280 nm detection for 22 min, t_R: 10.5 min). ^{1}H NMR (400 MHz) δ 2.35 (s, 3H), 3.56 (s, 2H), 3.74 (d, *J* = 9.2, 2H), 5.31 (t, *J* = 9.2, 1H), 7.45 (m, 3H), 7.64 (dd, *J* = 8.8, 2.0, 1H), 7.71 (m, 2H), 7.77- 7.82 (m, 2H), 10.69 (s, 1H). HRMS-FAB (*m/z*): $[M + H]^+$ calcd for $C_{22}H_{19}N_2O_6$, 407.1243; found, 407.1233.

3-(2-Methyl)phenyl-isoxazoline AMCA substrate 5b (CSVI144N). The substrate was synthesized according to the general procedure for the preparation of 3-substituted isoxazoline-AMCA substrates (*vida supra*) and purified using preparatory C18 reversed-phase HPLC (CH_3CN/H_2O-0.1% TFA, 5-95% for 50 min, 8 mL/min, 220/254/280 nm detection for 70 min, t_R: 36.7 min). The substrate was ≥99% pure as determined by HPLC-MS analysis (CH_3CN/H_2O-0.1% TFA, 5-95% for 14 min, 0.4 mL/min, 220/254/280 nm detection for 22 min, t_R: 11.0 min). ^{1}H NMR (400 MHz) δ 2.39 (s, 3H), 2.49 (s, 3H), 3.60 (s, 2H), 3.80-3.87 (m, 2H), 5.29 (dd, *J* = 9.8, 8.2, 1H), 7.30-7.39 (m, 3H), 7.53 (d, *J* = 7.5, 1H), 7.68 (dd, *J* = 8.7, 1.6, 1H), 7.81-7.86 (m, 2H), 10.73 (s, 1H). HRMS-FAB (*m/z*): $[M + H]^+$ calcd for $C_{23}H_{21}N_2O_6$, 421.1400; found, 421.1397.

3-(3-Methyl)phenyl-isoxazoline AMCA substrate 5c (CSVI144a). The substrate was synthesized according to the general procedure for the preparation of 3-substituted isoxazoline-AMCA substrates (*vida supra*) and purified using preparatory C18 reversed-phase HPLC (CH_3CN/H_2O-0.1% TFA, 5-95% for 50 min, 8 mL/min, 220/254/280 nm detection for 70 min, t_R: 36.7 min). The substrate was ≥99% pure as determined by HPLC-MS analysis (CH_3CN/H_2O-0.1% TFA, 5-95% for 14 min, 0.4 mL/min, 220/254/280 nm detection for 22 min, t_R: 11.2 min). ^{1}H NMR (400 MHz) δ 2.36 (s, 3H), 2.38 (s, 3H), 3.60 (s, 2H), 3.75 (m, 2H), 5.34 (t, *J* = 10.0, 1H), 7.30-7.39 (m, 2H), 7.52-7.56 (m, 2H),7.67 (dd, *J* = 8.4, 1.6, 1H), 7.80-7.85 (m, 2H), 10.71 (s, 1H). HRMS-FAB (*m/z*): $[M + H]^+$ calcd for $C_{23}H_{21}N_2O_6$, 421.1400; found, 421.1393.

3-(4-Methyl)phenyl-isoxazoline AMCA substrate 5d (CSVI144D). The substrate was synthesized according to the general procedure for the preparation of 3-substituted isoxazoline-AMCA substrates (*vida supra*) and purified using preparatory C18 reversed-phase HPLC (CH_3CN/H_2O-0.1% TFA, 5-95% for 50 min, 8 mL/min, 220/254/280 nm detection for 70 min, t_R: 36.7 min). The substrate was ≥99% pure as determined by HPLC-MS analysis (CH_3CN/H_2O-0.1% TFA, 5-95% for 14 min, 0.4 mL/min, 220/254/280 nm detection for 22 min, t_R: 10.4 min). ^{1}H NMR (400 MHz) δ 2.38 (s, 3H), 2.38 (s, 3H), 3.60 (s, 2H), 3.75 (d, *J* = 9.6, 2H), 5.32 (t, *J* = 9.2, 1H), 7.29 (d, *J* = 7.6, 2H), 7.62-7.69 (m, 3H), 7.80-7.85 (m, 2H), 10.70 (s, 1H). HRMS-FAB (*m/z*): $[M + H]^+$ calcd for $C_{23}H_{21}N_2O_6$, 421.1400; found, 421.1389.

3-(2-Fluoro)phenyl-isoxazoline AMCA substrate 5e (CSVI144c). The substrate was synthesized according to the general procedure for the preparation of 3-substituted isoxazoline-AMCA substrates (*vida supra*) and purified using preparatory C18 reversed-phase HPLC (CH_3CN/H_2O-0.1% TFA, 5-95% for 50 min, 8 mL/min, 220/254/280 nm detection for 70 min, t_R: 34.7 min). The substrate was ≥99% pure as determined by HPLC-MS analysis (CH_3CN/H_2O-0.1% TFA, 5-95% for 14 min, 0.4 mL/min, 220/254/280 nm detection for 22 min, t_R: 9.8 min). ^{1}H NMR (400 MHz) δ 2.39 (s, 3H), 3.60 (s, 2H), 3.80 (d, *J* = 9.2, 2H), 5.35 (t, *J* = 9.2, 1H), 7.30-7.39 (m, 2H), 7.68 (m, 1H), 7.67 (dd, *J* = 8.8, 2.0, 1H), 7.78-7.85 (m, 3H), 10.74 (s, 1H). HRMS-FAB (*m/z*): $[M + H]^+$ calcd for $C_{22}H_{18}FN_2O_6$, 425.1149; found, 425.1154.

3-(3-Fluoro)phenyl-isoxazoline AMCA substrate 5f (CSVI144H). The substrate was synthesized according to the general procedure for the preparation of 3-substituted isoxazoline-AMCA substrates (*vida supra*) and purified using preparatory C18 reversed-phase HPLC (CH_3CN/H_2O-0.1% TFA, 5-95% for 50 min, 8 mL/min, 220/254/280 nm detection for 70 min, t_R: 35.6 min). The substrate was ≥99% pure as determined by HPLC-MS analysis (CH_3CN/H_2O-0.1% TFA, 5-95% for 14 min, 0.4 mL/min, 220/254/280 nm detection for 22 min, t_R: 10.7 min). ^{1}H NMR (400 MHz) δ 2.39 (s, 3H), 3.60 (s, 2H), 3.78 (d, *J* = 10.0, 2H), 5.38 (t, *J* = 9.6, 1H), 7.36 (t, *J* = 5.6, 1H), 7.55-7.61 (m, 3H), 7.67 (d, *J* = 9.2, 1H), 7.80-7.85 (m, 2H), 10.73 (s, 1H). HRMS-FAB (*m/z*): $[M + H]^+$ calcd for $C_{22}H_{18}FN_2O_6$, 425.1149; found, 425.1154.

3-(4-Fluoro)phenyl-isoxazoline AMCA substrate 5g (CSVI144P). The substrate was synthesized according to the general procedure for the preparation of 3-substituted isoxazoline-AMCA substrates (*vida supra*) and purified using preparatory C18 reversed-phase HPLC (CH_3CN/H_2O-0.1% TFA, 5-95% for 50 min, 8 mL/min, 220/254/280 nm detection for 70 min, t_R: 35.2 min). The substrate was ~99% pure as determined by HPLC-MS analysis (CH_3CN/H_2O-0.1% TFA, 5-95% for 14 min, 0.4 mL/min, 220/254/280 nm detection for 22 min, t_R: 10.8 min). ^{1}H NMR (400 MHz) δ 2.38 (s, 3H), 3.60 (s, 2H), 3.77 (d, *J* = 10.0, 2H), 5.35 (dd, *J* = 9.6, 8.4, 1H), 7.33 (t, *J* = 8.8, 2H), 7.67 (dd, *J* = 9.2, 2.4, 1H), 7.78-7.85 (m, 4H), 10.71 (s, 1H). HRMS-FAB (*m/z*): $[M + H]^+$ calcd for $C_{22}H_{18}FN_2O_6$, 425.1149; found, 425.1154.

3-Cyclohexyl-isoxazoline AMCA substrate 5h (CSVI144B). The substrate was synthesized according to the general procedure for the preparation of 3-substituted isoxazoline-AMCA substrates (*vida supra*) and purified using preparatory C18 reversed-phase HPLC (CH_3CN/H_2O-0.1% TFA, 5-95% for 50 min, 8 mL/min, 220/254/280 nm detection for 70 min, t_R: 36.7 min). The substrate was ~99% pure as determined by HPLC-MS analysis (CH_3CN/H_2O-0.1% TFA, 5-95% for 14 min, 0.4 mL/min, 220/254/280 nm detection for 22 min, t_R: 10.7 min). ^{1}H NMR (400 MHz) δ 1.24-1.30 (m, 6H), 1.62-1.82 (m, 6H), 2.38 (m, 4H), 3.59 (s, 2H), 5.08 (t, *J* = 8.7, 1H), 7.66 (d, *J* = 8.6, 1H), 7.78-7.84 (m, 2H), 10.56 (s, 1H). HRMS-FAB (*m/z*): $[M + H]^+$ calcd for $C_{22}H_{25}N_2O_6$, 413.1713; found, 413.1707.

3-(Hydrocinnamyl)phenyl-isoxazoline AMCA substrate 5i (CSVI144O). The substrate was synthesized according to the general procedure for the preparation of 3-substituted isoxazoline-AMCA substrates (*vida supra*) and purified using preparatory C18 reversed-phase HPLC (CH_3CN/H_2O-0.1% TFA, 5-95% for 50 min, 8 mL/min, 220/254/280 nm detection for 70 min, t_R: 35.9 min). The substrate was ~99% pure as determined by HPLC-MS analysis (CH_3CN/H_2O-0.1% TFA, 5-95% for 14 min, 0.4 mL/min, 220/254/280 nm detection for 22 min, t_R: 10.5 min). ^{1}H NMR (400 MHz) δ 2.50 (s, 3H), 2.64-2.72 (m, 2H), 2.87 (t, *J* = 7.6, 2H), 3.60 (s, 2H), 5.11 (dd, *J* = 10.0, 8.0, 1H), 7.18 (m, 5H), 7.68 (dd, *J* = 8.4, 1.6, 1H), 7.80-7.86 (m, 2H), 10.53 (s, 1H). HRMS-FAB (*m/z*): $[M + H]^+$ calcd for $C_{24}H_{23}N_2O_6$, 435.1556; found, 435.1154.

3-Cinnamyl-isoxazoline AMCA substrate 5j (CSVI144I). The substrate was synthesized according to the general procedure for the preparation of 3-substituted isoxazoline-AMCA substrates (*vida supra*) and purified using preparatory C18 reversed-phase HPLC (CH_3CN/H_2O-0.1% TFA, 5-95% for 50 min, 8 mL/min, 220/254/280 nm detection for 70 min, t_R: 37.1 min). The substrate was ~99% pure as determined by HPLC-MS analysis (CH_3CN/H_2O-0.1% TFA, 5-95% for 14 min, 0.4 mL/min, 220/254/280 nm detection for 22 min, t_R: 10.2 min). ^{1}H NMR (400 MHz) δ 2.38 (s, 3H), 3.60-3.67 (m, 4H), 5.30 (dd, *J* = 10.8, 7.2, 1H), 7.13 (dd, *J* = 30.8, 16.4, 2H), 7.33-7.43 (m, 3H), 7.64-7.80 (m, 3H), 7.82-7.85 (m, 2H), 10.69 (s, 1H). HRMS-FAB (*m/z*): $[M + H]^+$ calcd for $C_{24}H_{21}N_2O_6$, 433.1400; found, 433.1397.

3-(4-Phenyl)phenyl-isoxazoline AMCA substrate 5k (CSVI144J). The substrate was synthesized according to the general procedure for the preparation of 3-substituted isoxazoline-AMCA substrates (*vida supra*) and purified using preparatory C18 reversed-phase HPLC (CH_3CN/H_2O-0.1% TFA, 5-95% for 50 min, 8 mL/min, 220/254/280 nm detection for 70 min, t_R: 41.2 min). The substrate was ~99% pure as determined by HPLC-MS analysis (CH_3CN/H_2O-0.1% TFA, 5-95% for 14 min, 0.4 mL/min, 220/254/280 nm detection for 22 min, t_R: 11.3 min). ^{1}H NMR (400 MHz) δ 2.39 (s, 3H), 3.60 (s, 2H), 3.81 (d, *J* = 9.6, 2H), 5.37 (t, *J* = 9.6, 1H), 7.41 (t, *J* = 7.6, 1H), 7.50 (t, *J* = 7.6, 2H), 7.68-7.86 (m, 9H), 10.73 (s, 1H). HRMS-FAB (*m/z*): $[M + H]^+$ calcd for $C_{28}H_{23}N_2O_6$, 483.1156; found, 483.1562.

3-(1-Naphthyl)-isoxazoline AMCA substrate 5l (CSVI144L). The substrate was synthesized according to the general procedure for the preparation of 3-substituted isoxazoline-AMCA substrates (*vida supra*) and purified using preparatory C18 reversed-phase HPLC (CH_3CN/H_2O-0.1% TFA, 5-95% for 50 min, 8 mL/min, 220/254/280 nm detection for 70 min, t_R: 39.1 min). The substrate was ~99% pure as determined by HPLC-MS analysis (CH_3CN/H_2O-0.1% TFA, 5-95% for 14 min, 0.4 mL/min, 220/254/280 nm detection for 22 min, t_R: 11.2 min). ^{1}H NMR (400 MHz) δ 2.39 (s, 3H), 3.60 (s, 2H), 3.99 (m, 2H), 5.38 (dd, *J* = 10.4, 7.2, 1H), 7.60-7.72 (m, 4H), 7.82-7.88 (m, 3H), 8.03-8.09 (m, 2H), 8.91 (d, *J* = 8.8, 1H), 10.80 (s, 1H). HRMS-FAB (*m/z*): $[M + H]^+$ calcd for $C_{26}H_{21}N_2O_6$, 457.1400; found, 457.1398.

3-(2-Naphthyl)-isoxazoline AMCA substrate 5m (CSVI144M). The substrate was synthesized according to the general procedure for the preparation of 3-substituted isoxazoline-AMCA substrates (*vida supra*) and purified using preparatory C18 reversed-phase HPLC (CH_3CN/H_2O-0.1% TFA, 5-95% for 50 min, 8 mL/min, 220/254/280 nm detection for 70 min, t_R: 38.6 min). The substrate was ~99% pure as determined by HPLC-MS analysis (CH_3CN/H_2O-0.1% TFA, 5-95% for 14 min, 0.4 mL/min, 220/254/280 nm detection for 22 min, t_R: 10.7 min). ^{1}H NMR (400 MHz) δ 2.39 (s, 3H), 3.60 (s, 2H), 3.89-3.92 (m, 2H), 5.41 (dd, *J* = 10.4, 7.6, 1H), 7.59-7.62 (m, 2H), 7.69 (d, *J* = 9.6, 1H), 7.81-7.86 (m, 2H), 7.92-8.24 (m, 4H), 8.31 (s, 1H), 10.76 (s, 1H). HRMS-FAB (*m/z*): $[M + H]^+$ calcd for $C_{26}H_{21}N_2O_6$, 457.1400; found, 457.1411.

3-(4-Trifluoromethyl)phenyl-isoxazoline AMCA substrate 5n (CSVI144E). The substrate was synthesized according to the general procedure for the preparation of 3-substituted isoxazoline-AMCA substrates (*vida supra*) and purified using preparatory C18 reversed-phase HPLC (CH_3CN/H_2O-0.1% TFA, 5-95% for 50 min, 8 mL/min, 220/254/280 nm detection for 70 min, t_R: 338.6 min). The substrate was ~99% pure as determined by HPLC-MS analysis (CH_3CN/H_2O-0.1% TFA, 5-95% for 14 min, 0.4 mL/min, 220/254/280 nm detection for 22 min, t_R: 11.4 min). ^{1}H NMR (400 MHz) δ 2.37 (s, 3H), 3.59 (s, 2H), 3.82 (d, *J* = 9.2, 2H), 5.41 (t, *J* = 9.2, 1H), 7.67 (dd, *J* = 8.8, 1.6, 2H), 7.80-7.97 (m, 6H), 10.75 (s, 1H). HRMS-FAB (*m/z*): $[M + H]^+$ calcd for $C_{23}H_{18}F_3N_2O_6$, 475.1117; found, 475.1115.

3-(4-Methoxy)phenyl-isoxazoline AMCA substrate 5o (CSVI144F). The substrate was synthesized according to the general procedure for the preparation of 3-substituted isoxazoline-AMCA substrates (*vida supra*) and purified using preparatory C18 reversed-phase HPLC (CH_3CN/H_2O-0.1% TFA, 5-95% for 50 min, 8 mL/min, 220/254/280 nm detection for 70 min, t_R: 34.6 min). The substrate was ~99% pure as determined by HPLC-MS analysis (CH_3CN/H_2O-0.1% TFA, 5-95% for 14 min, 0.4 mL/min, 220/254/280 nm detection for 22 min, t_R: 10.0 min). ^{1}H NMR (400 MHz) δ 2.38 (s, 3H), 3.62 (s, 2H), 3.74 (d, *J* = 9.6, 2H), 3.81 (s, 3H), 5.30 (t, *J* = 9.6, 1H), 7.03 (d, *J* = 8.8, 2H), 7.68 (m, 3H), 7.80-7.86 (m, 2H), 10.68 (s, 1H). HRMS-FAB (*m/z*): $[M + H]^+$ calcd for $C_{23}H_{21}N_2O_7$, 437.1349; found, 437.1353.

3-(2-Methoxy)phenyl-isoxazoline AMCA substrate 5p (CSVI154I). The substrate was synthesized according to the general procedure for the preparation of 3-substituted isoxazoline-AMCA substrates (*vida supra*) and purified using preparatory C18 reversed-phase HPLC (CH_3CN/H_2O-0.1% TFA, 5-95% for 50 min, 8 mL/min, 220/254/280 nm detection for 70 min, t_R: 34.5 min). The substrate was ~99% pure as determined by HPLC-MS analysis (CH_3CN/H_2O-0.1% TFA, 5-95% for 14 min, 0.4 mL/min, 220/254/280 nm detection for 22 min, t_R: 9.3 min). ^{1}H NMR (400 MHz) δ 2.39 (s, 3H), 3.60 (s, 2H), 3.76 (d, *J* = 10.4, 2H), 3.86 (s, 3H), 5.28 (dd, *J* = 10.0, 8.0, 1H), 7.01 (t, *J* = 7.2, 1H), 7.15 (dd, *J* = 8.4, 1H), 7.47 (t, *J* = 7.2, 1H), 7.63-7.70 (m, 2H), 7.80-7.86 (m, 2H), 10.67 (s, 1H). HRMS-FAB (*m/z*): $[M + H]^+$ calcd for $C_{23}H_{21}N_2O_7$, 437.1349; found, 437.1340.

3-(2,3,4,5,6-Pentafluoro)phenyl-isoxazoline AMCA substrate 5q (CSVI154A). The substrate was synthesized according to the general procedure for the preparation of 3-substituted isoxazoline-AMCA substrates (*vida supra*) and purified using preparatory C18 reversed-phase HPLC (CH_3CN/H_2O-0.1% TFA, 5-95% for 50 min, 8 mL/min, 220/254/280 nm detection for 70 min, t_R: 37.4 min). The substrate was ~99% pure as determined by HPLC-MS analysis (CH_3CN/H_2O-0.1% TFA, 5-95% for 14 min, 0.4 mL/min, 220/254/280 nm detection for 22 min, t_R: 11.3 min). ^{1}H NMR (400 MHz) δ 2.39 (s, 3H), 3.60 (s, 2H), 3.79 (d, *J* = 9.2, 2H), 5.43 (t, *J* = 9.2, 1H), 7.66 (dd, *J* = 8.8, 2.0, 1H), 7.82-7.85 (m, 2H), 10.81 (s, 1H). HRMS-FAB (*m/z*): $[M + H]^+$ calcd for $C_{22}H_{14}F_5N_2O_6$, 497.0772; found, 497.0784.

3-(2-Chloro, 6-fluoro)phenyl-isoxazoline AMCA substrate 5r (CSVI154B). The substrate was synthesized according to the general procedure for the preparation of 3-substituted isoxazoline-AMCA substrates (*vida supra*) and purified using preparatory C18 reversed-phase HPLC (CH_3CN/H_2O-0.1% TFA, 5-95% for 50 min, 8 mL/min, 220/254/280 nm detection for 70 min, t_R: 35.7 min). The substrate was ~99% pure as determined by HPLC-MS analysis (CH_3CN/H_2O-0.1% TFA, 5-95% for 14 min, 0.4 mL/min, 220/254/280 nm detection for 22 min, t_R: 10.5 min). ^{1}H NMR (400 MHz) δ 2.39 (s, 3H), 3.60 (s, 2H), 3.71 (m, 2H), 5.46 (dd, *J* = 10.4, 7.2, 1H), 7.41 (t, *J* = 9.2, 1H), 7.49 (d, *J* = 8.0, 1H), 7.59 (dd, *J* = 14.4, 4.4, 1H), 7.68 (dd, *J* = 8.8, 1.6, 1H), 7.81-7.86 (m, 2H), 10.76 (s, 1H). HRMS-FAB (*m/z*): $[M + H]^+$ calcd for $C_{22}H_{17}ClFN_2O_6$, 459.0759; found, 459.0758.

3-(2,6-Difluoro)phenyl-isoxazoline AMCA substrate 5s (CSVI154C). The substrate was synthesized according to the general procedure for the preparation of 3-substituted isoxazoline-AMCA substrates (*vida supra*) and purified using preparatory C18 reversed-phase HPLC (CH_3CN/H_2O-0.1% TFA, 5-95% for 50 min, 8 mL/min, 220/254/280 nm detection for 70 min, t_R: 34.4 min). The substrate was ~99% pure as determined by HPLC-MS analysis (CH_3CN/H_2O-0.1% TFA, 5-95% for 14 min, 0.4 mL/min, 220/254/280 nm detection for 22 min, t_R: 9.5 min). ^{1}H NMR (400 MHz) δ 2.39 (s, 3H), 3.60 (s, 2H), 3.76 (d, *J* = 9.2, 2H), 5.37 (t, *J* = 9.6, 1H), 7.27 (t, *J* = 8.8, 2H), 7.58-7.69 (m, 2H), 7.81-7.85 (m, 2H), 10.77 (s, 1H). HRMS-FAB (*m/z*): $[M + H]^+$ calcd for $C_{22}H_{17}F_2N_2O_6$, 443.1055; found, 443.1050.

3-(2,4-Difluoro)phenyl-isoxazoline AMCA substrate 5t (CSVI154D). The substrate was synthesized according to the general procedure for the preparation of 3-substituted isoxazoline-AMCA substrates (*vida supra*) and purified using preparatory C18 reversed-phase HPLC (CH_3CN/H_2O-0.1% TFA, 5-95% for 50 min, 8 mL/min, 220/254/280 nm detection for 70 min, t_R: 35.5 min). The substrate was ~99% pure as determined by HPLC-MS analysis (CH_3CN/H_2O-0.1% TFA, 5-95% for 14 min, 0.4 mL/min, 220/254/280 nm detection for 22 min, t_R: 10.2 min). ^{1}H NMR (400 MHz) δ 2.38 (s, 3H), 3.59 (s, 2H), 3.79 (d, *J* = 8.0, 2H), 5.34 (t, *J* = 9.2, 1H), 7.23 (t, *J* = 8.0, 1H), 7.44 (td, *J* = 12.0, 2.4, 1H), 7.67 (dd, *J* = 8.8, 2.0, 1H), 7.81-7.89 (m, 3H), 10.73 (s, 1H). HRMS-FAB (*m/z*): $[M + H]^+$ calcd for $C_{22}H_{17}F_2N_2O_6$, 443.1055; found, 443.1055.

3-(2,5-Difluoro)phenyl-isoxazoline AMCA substrate 5u (CSVI154E). The substrate was synthesized according to the general procedure for the preparation of 3-substituted isoxazoline-AMCA substrates (*vida supra*) and purified using preparatory C18 reversed-phase HPLC (CH_3CN/H_2O-0.1% TFA, 5-95% for 50 min, 8 mL/min, 220/254/280 nm detection for 70 min, t_R: 35.5 min). The substrate was ~99% pure as determined by HPLC-MS analysis (CH_3CN/H_2O-0.1% TFA, 5-95% for 14 min, 0.4 mL/min, 220/254/280 nm detection for 22 min, t_R: 10.3 min). ^{1}H NMR (400 MHz) δ 2.39 (s, 3H), 3.60 (s, 2H), 3.80 (d, *J* = 9.2, 2H), 5.37 (t, *J* = 9.2, 1H), 7.41-7.45 (m, 2H), 7.60-7.68 (m, 2H), 7.81-7.85 (m, 2H), 10.74 (s, 1H). HRMS-FAB (*m/z*): $[M + H]^+$ calcd for $C_{22}H_{17}F_2N_2O_6$, 443.1055; found, 443.1062.

3-(2-Fluoro, 3-trifluoromethyl)phenyl-isoxazoline AMCA substrate 5v (CSVI154F). The substrate was synthesized according to the general procedure for the preparation of 3-substituted isoxazoline-AMCA substrates (*vida supra*) and purified using preparatory C18 reversed-phase HPLC (CH_3CN/H_2O-0.1% TFA, 5-95% for 50 min, 8 mL/min, 220/254/280 nm detection for 70 min, t_R: 38.5 min). The substrate was ~99% pure as determined by HPLC-MS analysis (CH_3CN/H_2O-0.1% TFA, 5-95% for 14 min, 0.4 mL/min, 220/254/280 nm detection for 22 min, t_R: 11.0 min). ^{1}H NMR (400 MHz) δ 2.38 (s, 3H), 3.58 (s, 2H), 3.85 (d, *J* = 10.0, 2H), 5.40 (t, *J* = 9.2, 1H), 7.53 (t, *J* = 6.8, 1H), 7.67 (d, *J* = 6.4, 1H), 7.81-7.85 (m, 2H), 7.93 (t, *J* = 7.6, 1H), 8.12 (t, *J* = 8.8, 1H), 10.75 (s, 1H). HRMS-FAB (*m/z*): $[M]^+$ calcd for $C_{23}H_{16}F_4N_2O_6$, 492.0944; found, 492.0945.

3-(2-Fluoro, 4-bromo)phenyl-isoxazoline AMCA substrate 5w (CSVI154G). The substrate was synthesized according to the general procedure for the preparation of 3-substituted isoxazoline-AMCA substrates (*vida supra*) and purified using preparatory C18 reversed-phase HPLC (CH_3CN/H_2O-0.1% TFA, 5-95% for 50 min, 8 mL/min, 220/254/280 nm detection for 70 min, t_R: 38.1 min). The substrate was ~99% pure as determined by HPLC-MS analysis (CH_3CN/H_2O-0.1% TFA, 5-95% for 14 min, 0.4 mL/min, 220/254/280 nm detection for 22 min, t_R: 11.4 min). ^{1}H NMR (400 MHz) δ 2.39 (s, 3H), 3.60 (s, 2H), 3.80 (d, *J* = 8.8, 2H), 5.37 (t, *J* = 9.2, 1H), 7.53 (dd, *J* = 10.8, 8.8, 1H), 7.66 (dd, *J* = 8.8, 2.0, 1H), 7.74 (q, *J* = 4.4, 1H), 7.81-7.85 (m, 2H), 7.93 (dd, *J* = 6.8, 2.8, 1H), 10.73 (s, 1H). HRMS-FAB (*m/z*): $[M + H]^+$ calcd for $C_{22}H_{17}BrFN_2O_6$, 503.0254; found, 503.0261.

3-(2-Fluoro, 6-trifluoromethyl)phenyl-isoxazoline AMCA substrate 5x (CSVI154H). The substrate was synthesized according to the general procedure for the preparation of 3-substituted isoxazoline-AMCA substrates (*vida supra*) and purified using preparatory C18 reversed-phase HPLC (CH_3CN/H_2O-0.1% TFA, 5-95% for 50 min, 8 mL/min, 220/254/280 nm detection for 70 min, t_R: 36.1 min). The substrate was ~99% pure as determined by HPLC-MS analysis (CH_3CN/H_2O-0.1% TFA, 5-95% for 14 min, 0.4 mL/min, 220/254/280 nm detection for 22 min, t_R: 10.1 min). ^{1}H NMR (400 MHz) δ 2.40 (s, 3H), 3.61 (s, 2H), 3.67 (m, 2H), 5.49 (dd, *J* = 10.4, 7.2, 1H), 7.68-7.85 (m, 6H), 10.73 (s, 1H). HRMS-FAB (*m/z*): $[M + H]^+$ calcd for $C_{23}H_{17}F_4N_2O_6$, 493.1023; found, 493.1020.

Screening of focused library of 3-substituted isoxazoline substrates. Substrate stock solutions were prepared in DMSO. The substrates were diluted in assay buffer (50 mM Tris pH 8.1, 1 mM EDTA, and 0.01% tween) to a final concentration of 10 μM. The concentration of DMSO in the assays was less than 5%. The final chymotrypsin concentration was 50 nM. The hydrolysis of AMCA substrates was monitored fluorometrically with an excitation wavelength of 370 nm and emission wavelength of 455 nm on a Fluoromax-2 spectrofluorimeter (Molecular Devices, Sunnyvale, CA). All individual rates were measured in duplicate at 37 ºC, and the rate of non-enzymatic background hydrolysis was subtracted.

Determination of K_ms for selected isoxazoline substrates. Individual kinetic constants were determined for selected good substrates. Substrate stock solutions were prepared in DMSO. The substrates were diluted in assay buffer (50 mM Tris pH 8.1, 1 mM EDTA, and 0.01% tween) to a final concentration ranging from 4 μM to 500 μM. The concentration of DMSO in the assays was less than 5%. The final chymotrypsin concentration was 50 nM. The hydrolysis of AMCA substrates was monitored fluorometrically with an excitation wavelength of 370 nm and emission wavelength of 455 nm on a Fluoromax-2 spectrofluorimeter (Molecular Devices, Sunnyvale, CA). Rates were measured at 37 ºC, and the non-enzymatic background hydrolysis was subtracted. The corrected rates were fit to the Michaelis-Menten equation for steady state kinetics in order to determine the K_ms. All individual rates were measured in duplicate.

Substrate	K_m [μM]
5a (CSVI132D)	130 ± 10
5e (CSVI144C)	100 ± 20
5f (CSVI144H)	84 ± 4

Vinyl phosphonic acid di-(3-chloro)phenyl ester 7 (CSVI201). Phosphonate **7** was synthesized following procedures similar to those reported by Okamoto.[3] To a flame-dried 100 mL round bottom flask fitted with a stir bar under N_2 were added sodium iodide

(3.9 g, 26 mmol), diethyl vinyl phosphonate **6** (2.0 mL, 13 mmol), trimethylchlorosilane (3.3 mL, 26 mmol), and acetonitrile (13 mL). The reaction mixture was stirred for 30 min, then heated to 40 ºC and stirred for an additional 30 min. The reaction mixture was then cooled to rt and filtered through a fine-mesh glass frit. The filtrate was concentrated to provide crude di(trimethylsilyl) vinyl phosphonate as a red liquid.

Into a flame-dried 100 mL round bottom flask fitted with a stir bar under N_2 were added PCl_5 (3.3 g ,16 mmol) and CH_2Cl_2 (8 mL). To this mixture was added the crude di(trimethylsilyl) vinyl phosphonate in CH_2Cl_2 (8 mL). The reaction mixture was allowed to stir for 30 min and concentrated to provide the crude vinyl phosphonic acid dichloride as a red liquid.

To the 100 mL round bottom flask containing the crude vinyl phosphonic acid dichloride was added *m*-chlorophenol (2.7 mL, 26 mmol). A reflux condenser was fitted to the round bottom flask containing the reaction mixture, and the reaction mixture was heated to 150 ºC for 8 h. The reaction mixture was cooled to RT and diluted with $CHCl_3$ (25 mL). The solution was washed with 20% NaOH (20 mL) and water (30 mL). The organic layer was dried ($MgSO_4$) and then concentrated. Purification over silica gel (EtOAc/hexanes 20:80) provided 595 mg (14 % over three steps) of vinyl phosphonic acid di-3-chlorophenyl ester **7** as a yellow oil. The compound was approx. 99% pure as determined by HPLC-MS analysis (CH_3CN/H_2O-0.1% TFA, 5-95% for 14 min, 0.8 mL/min, 220/254/280 nm detection for 22 min, t_R: 13.6 min). ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.17-6.63 (m, 3H), 7.10-7.29 (m, 8H). ^{13}C (400 MHz, $CDCl_3$) δ 118.9 (d, *J* = 17.6), 121.2 (d, *J* = 19.2), 124.0 (d, *J* = 743.6), 125.8, 130.6, 135.1, 139.4, 150.4 (d, *J* = 30.4). ^{31}P (400 MHz, $CDCl_3$) δ 10.57. HRMS-FAB (*m/z*): $[M + H]^+$ calcd for $C_{14}H_{12}O_3PCl_2$, 328.9901; found, 328.9910.

[3-(3-Fluorophenyl)-4,5-dihydro-isoxazol-5-yl]phosphonic acid di-(3-chloro)phenyl ester 8 (CSVII49f). Inhibitor **8** was synthesized following procedures similar to those reported by Okamoto.[4] To a flame-dried 10 mL pear-shaped flask fitted with a stir bar under N_2 was added crude 3-fluorobenzaldehyde oxime (*vida supra*) (27.8 mg, 0.200 mmol), *N*-chlorosuccinimide (27.0 g, 0.200 mmol), and anhydrous DMF (1 mL). The fumes from a bottle of concentrated HCl were bubbled through the solution until the solution turned slightly yellow. After stirring for 1.5 h, a solution of phosphonate **7** (66.0 mg, 0.200 mmol) and *i*-Pr_2NEt (35 μL, 0.20 mmol) in CH_2Cl_2 (200 μL) was added. The reac-

tion mixture was stirred for 1.5 h and was then concentrated using a rotary evaporator. EtOAc (2 mL) was added to the residue, and the resulting solution was washed with water (3 x 1 mL) and brine (1 x 1 mL). The organic layer was dried with $MgSO_4$, filtered, and concentrated. Purification over silica gel (25:75 EtOAc/hexanes) provided 20.4 mg (22 %) of inhibitor **8** as a clear oil. The compound was ~99% pure as determined by HPLC-MS analysis (CH_3CN/H_2O-0.1% TFA, 5-95% for 14 min, 0.8 mL/min, 220/254/280 nm detection for 22 min, t_R: 14.7 min). ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.71-3.90 (m, 2H), 5.22 (ddd, *J* = 11.6, 9.6, 4.0, 1H), 7.13-7.43 (m, 12H). ^{13}C (400 MHz, $CDCl_3$) δ 37.6, 74.4 (d, *J* = 169.0), 113.9 (d, *J* = 23.3), 117.9 (d, *J* = 21.4), 118.9 (dd, *J* = 56.2, 15.4), 121.2 (dd, *J* = 58.8, 17.8), 122.8 (d, *J* = 51.6), 126.2 (d, *J* = 70.6), 130.0, 130.6 (d, *J* = 24.0), 130.7 (d, *J* = 56.0), 135.2 (d, *J* = 48.9), 150.1 (dd, *J* = 115.4, 36.9), 161.6, 164.0. ^{31}P (400 MHz, $CDCl_3$) δ 11.86. HRMS-FAB (*m/z*): $[M + H]^+$ calcd for $C_{21}H_{16}O_4PFNCl_2$, 466.0178; found, 466.0174.

Methyl phosphonic acid di-(3-chloro)phenyl ester 10 (CSVII6). To a flame-dried 25 mL round bottom flask fitted with a stir bar and reflux condenser under N_2 was added *m*-chlorophenol (1.3 mL, 13 mmol) and methyl phosphonic dichloride (806 mg, 6.00 mmol). The reaction mixture was heated to 120 ºC for 8 h. The reaction mixture was allowed to cool to rt, and $CHCl_3$ (25 mL) was added. The solution was washed with 10% NaOH (20 mL) and water (30 mL). The organic layer was dried with $MgSO_4$, filtered, and concentrated. Purification over silica gel (35:65 EtOAc/hexanes) provided 992 mg (52 %) of the minimal warhead **10** as a yellow oil. The compound was ~99% pure as determined by HPLC-MS analysis (CH_3CN/H_2O-0.1% TFA, 5-95% for 14 min, 0.8 mL/min, 220/254/280 nm detection for 22 min, t_R: 12.2 min). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.83 (d, *J* = 17.7, 3H), 7.11-7.30 (m, 8H). ^{13}C (400 MHz, $CDCl_3$) δ 11.6 (d, *J* = 576.0), 118.8 (d, *J* = 16.0), 121.1 (d, *J* = 16.0), 125.8, 130.6, 150.5. ^{31}P (400 MHz, $CDCl_3$) δ 24.42. HRMS-FAB (*m/z*): $[M + H]^+$ calcd for $C_{13}H_{12}O_3PCl_2$, 316.9901; found, 316.9907.

Progress curve method for determining k_{inact}/K_i. The k_{inact}/K_i for inhibitor **8** and minimal warhead **10** were determined under pseudo-first order conditions using the progress curve method.[5] Assay wells contained a mixture of inhibitor (0–1 μM for **8** and 0-120 μM for **10**) and 250 μM Suc-AAPF-AMC in buffer (50 mM Tris pH 8.1, 1 mM EDTA, and 0.01% tween) at 37 °C. Aliquots of chymotrypsin were added to each well to initiate the

assay. The final enzyme concentration was 25 nM. Hydrolysis of the AMC substrates was monitored fluorometrically for 45 min with an excitation wavelength of 380 nm and emission wavelength of 460 nm on a Fluoromax-2 spectrofluorimeter (Molecular Devices, Sunnyvale, CA). To determine the inhibition parameters, time points for which the control ([I] = 0) is linear were used. For each inhibitor, a k_{obs} was calculated for at least four different concentrations of inhibitors via a nonlinear regression of the data according to the equation

$$P = (v_i / k_{obs})[1 - \exp(-k_{obs} t)]$$

(where product formation = P, initial rate = v_i, time = t, and the first-order rate constant = k_{obs}.). The determined k_{obs} can be applied to the equation

$$(1/ k_{obs}) = (K_i/k_{inact})(1/ [I]) + 1/ k_{inact}$$

which states the K_i/k_{inact} of each inhibitor is equal to the slope of $1/k_{obs}$ vs. 1/inhibitor concentration [I]. Inhibition was measured in quadruplicate, and the average of the four assays is reported.

Protease selectivity assay. Assay wells containing enzyme in buffer (control) or enzyme and 1 μM inhibitor **8** in buffer were incubated at 37 ºC for 5 min, and substrate added to initiate the assay. Inhibitor and substrate stock solutions were prepared in DMSO. The assay buffer for all enzymes was 50 mM Tris pH 8.1, 1 mM EDTA, and 0.01% Tween. For chymotrypsin, the final substrate (Suc-AAPF-AMC) concentration was 5 μM and the final enzyme concentration was 1.25 nM. For trypsin, the final substrate (Z-FR-AMC) concentration was 5 μM and the final enzyme concentration was 25 nM. For elastase, the final substrate (MeOSuc-AAPV-AMC) concentration was 25 μM and the final enzyme concentration was 25 nM. For cathepsin G, the final substrate (Suc-AAPF-AMC) concentration was 25 μM and the final enzyme concentration was 250 nM. The concentration of DMSO in the assays was less than 6%. Hydrolysis of AMC substrates was

monitored fluorometrically with an excitation wavelength of 380 nm and emission wavelength of 460 nm on a Fluoromax-2 spectrofluorimeter (Molecular Devices, Sunnyvale, CA). All individual rates were measured in triplicate, and the percent activity calculated by the equation:

% activity = (average rate of the inhibited enzymes/average rate of the control) x 100.

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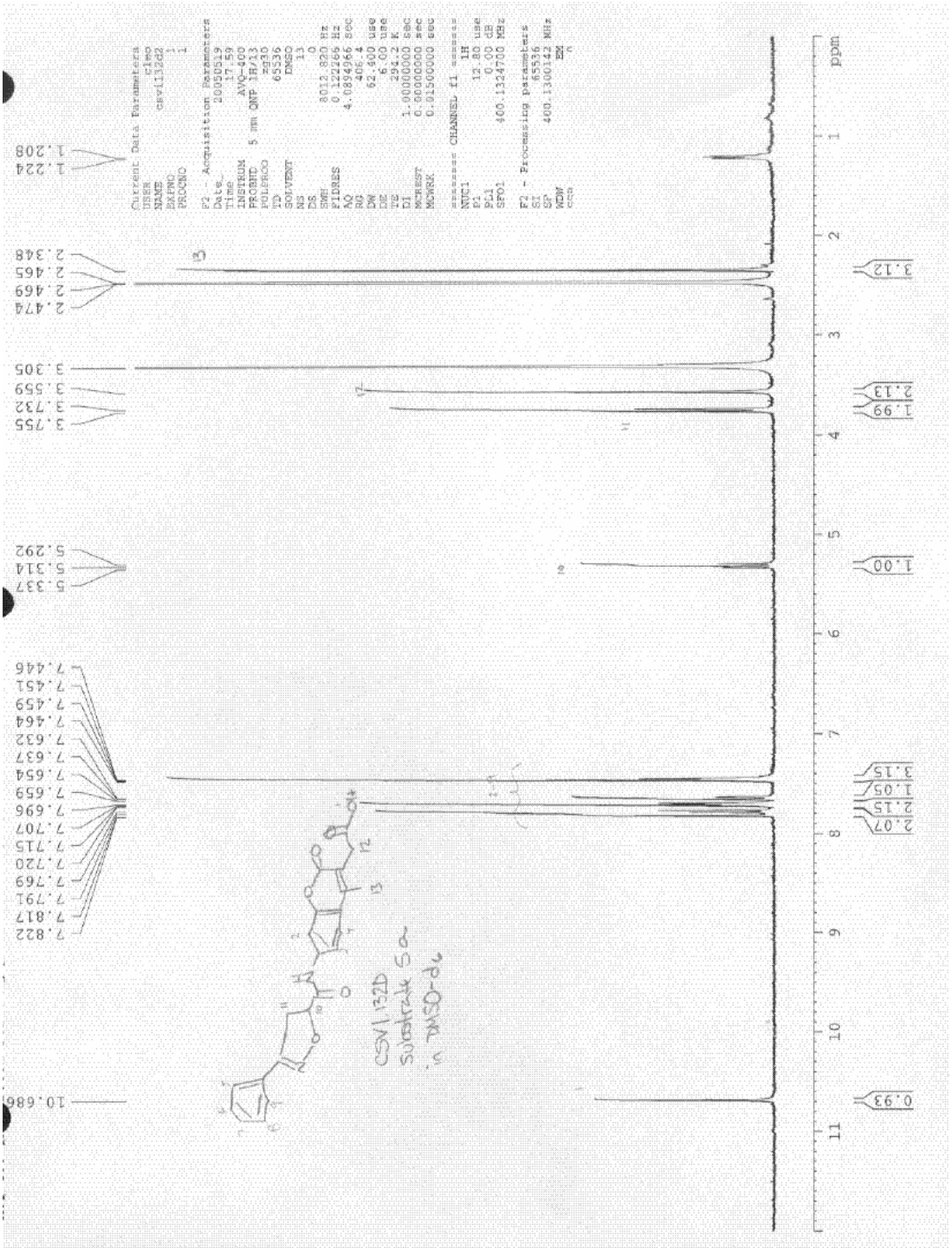
Substrate **5a**



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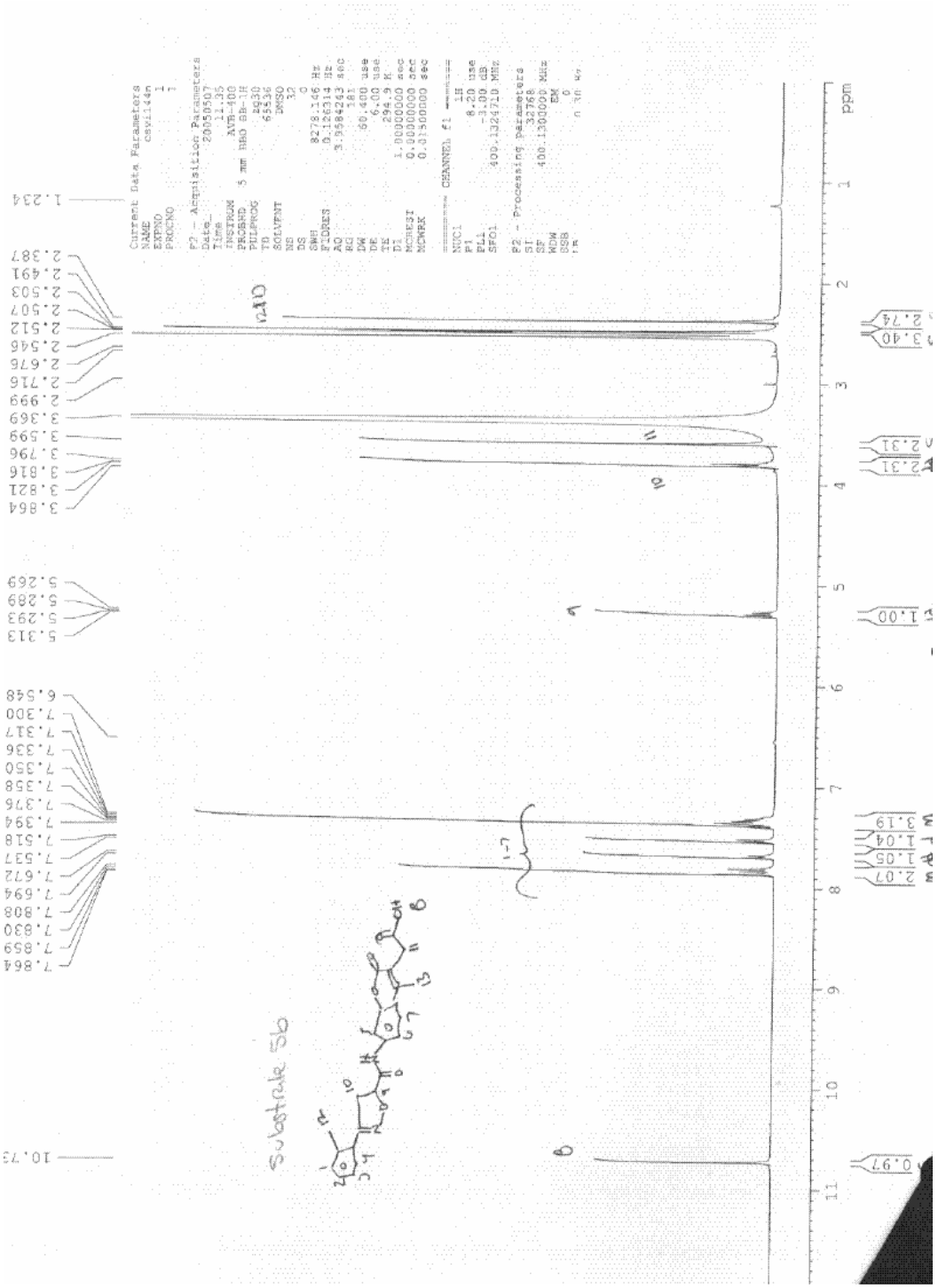

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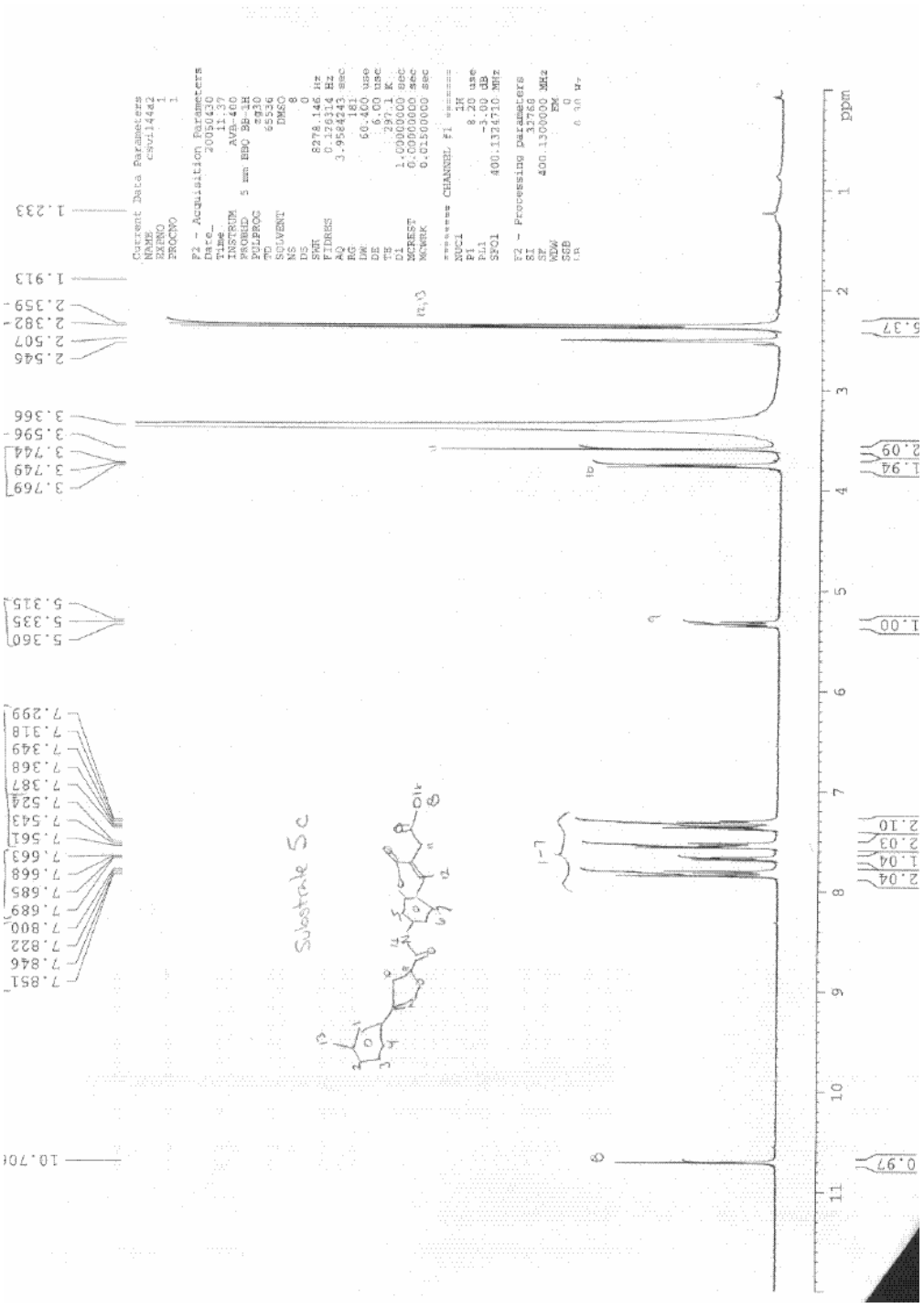

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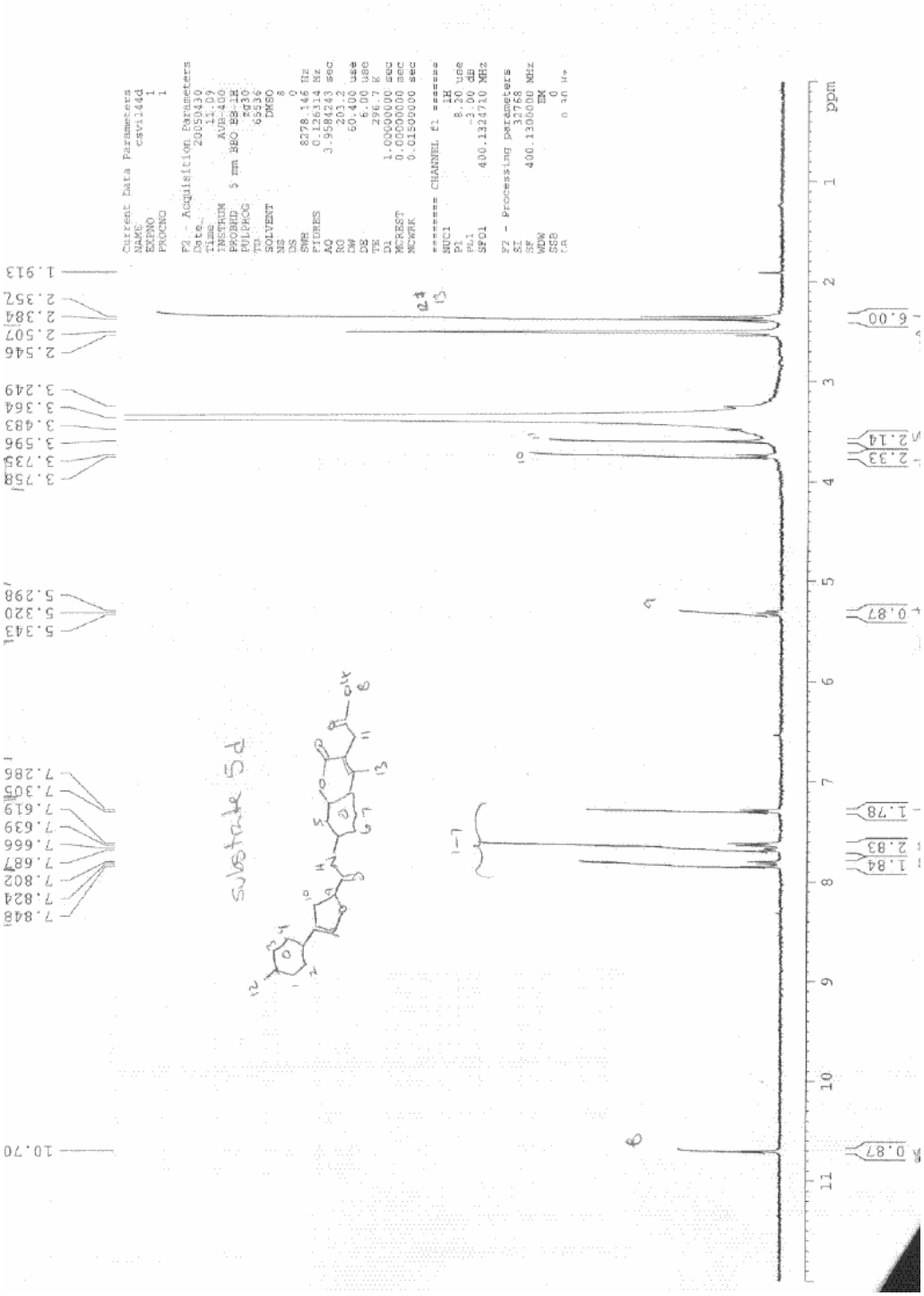

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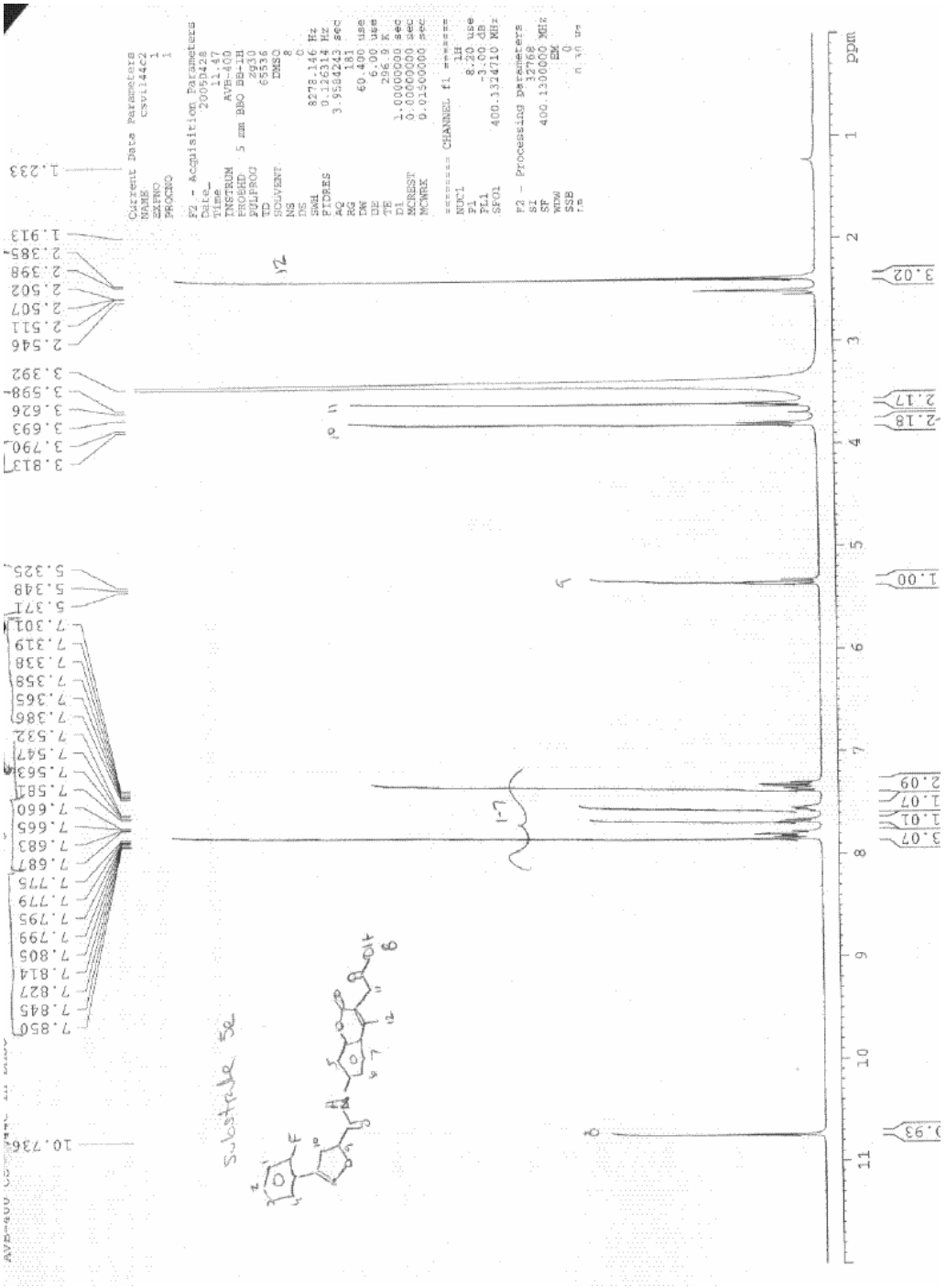

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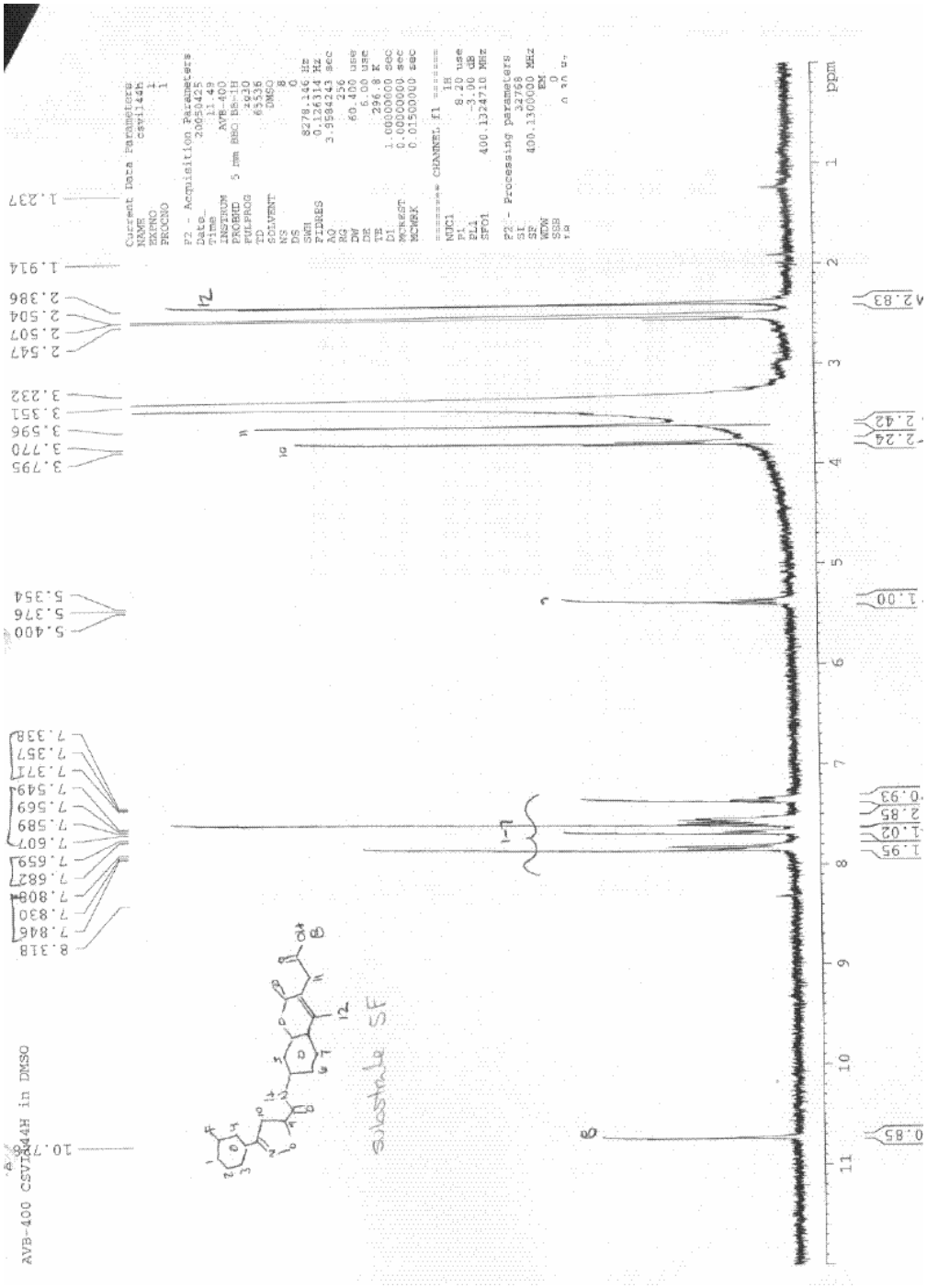

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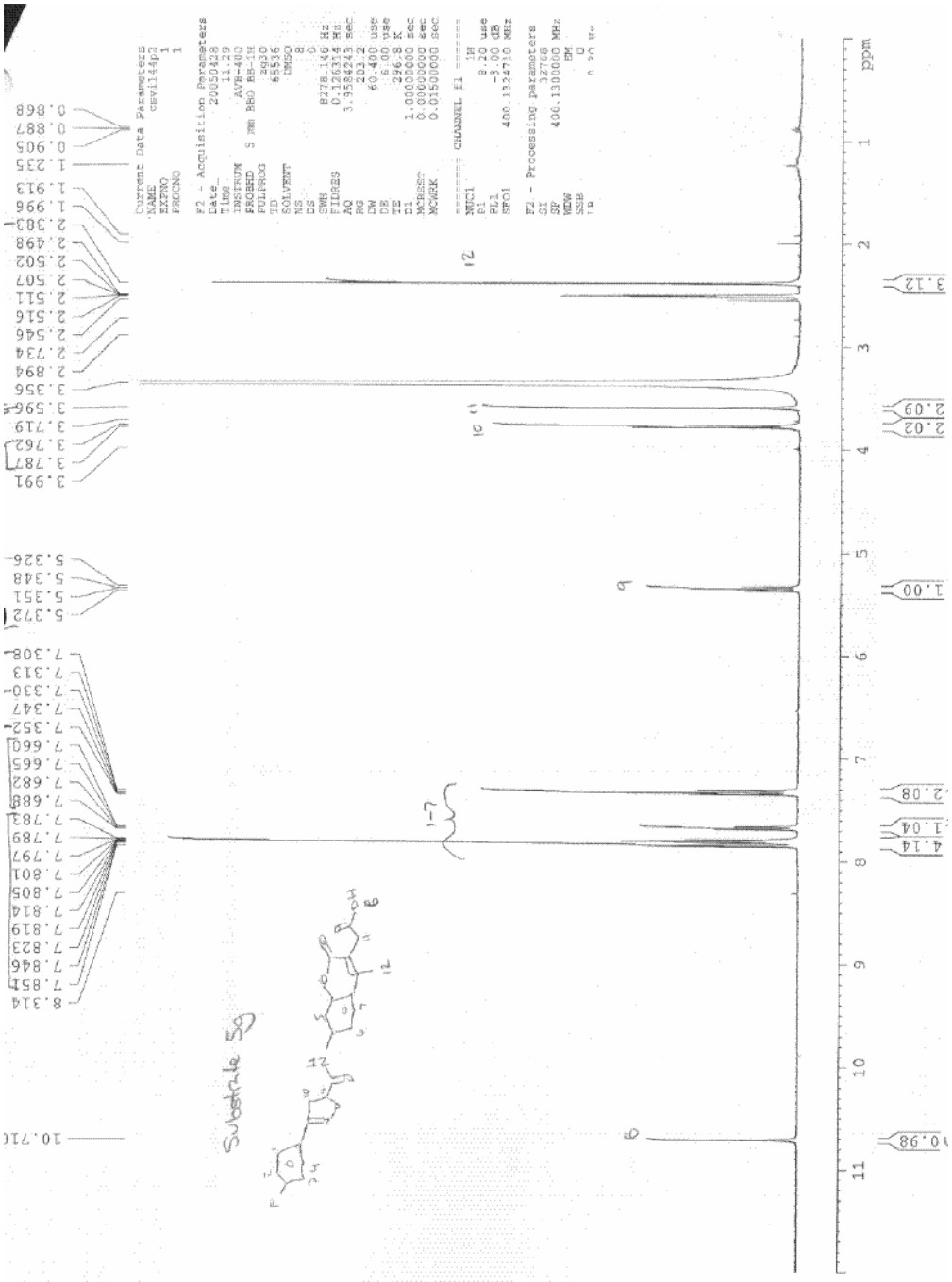

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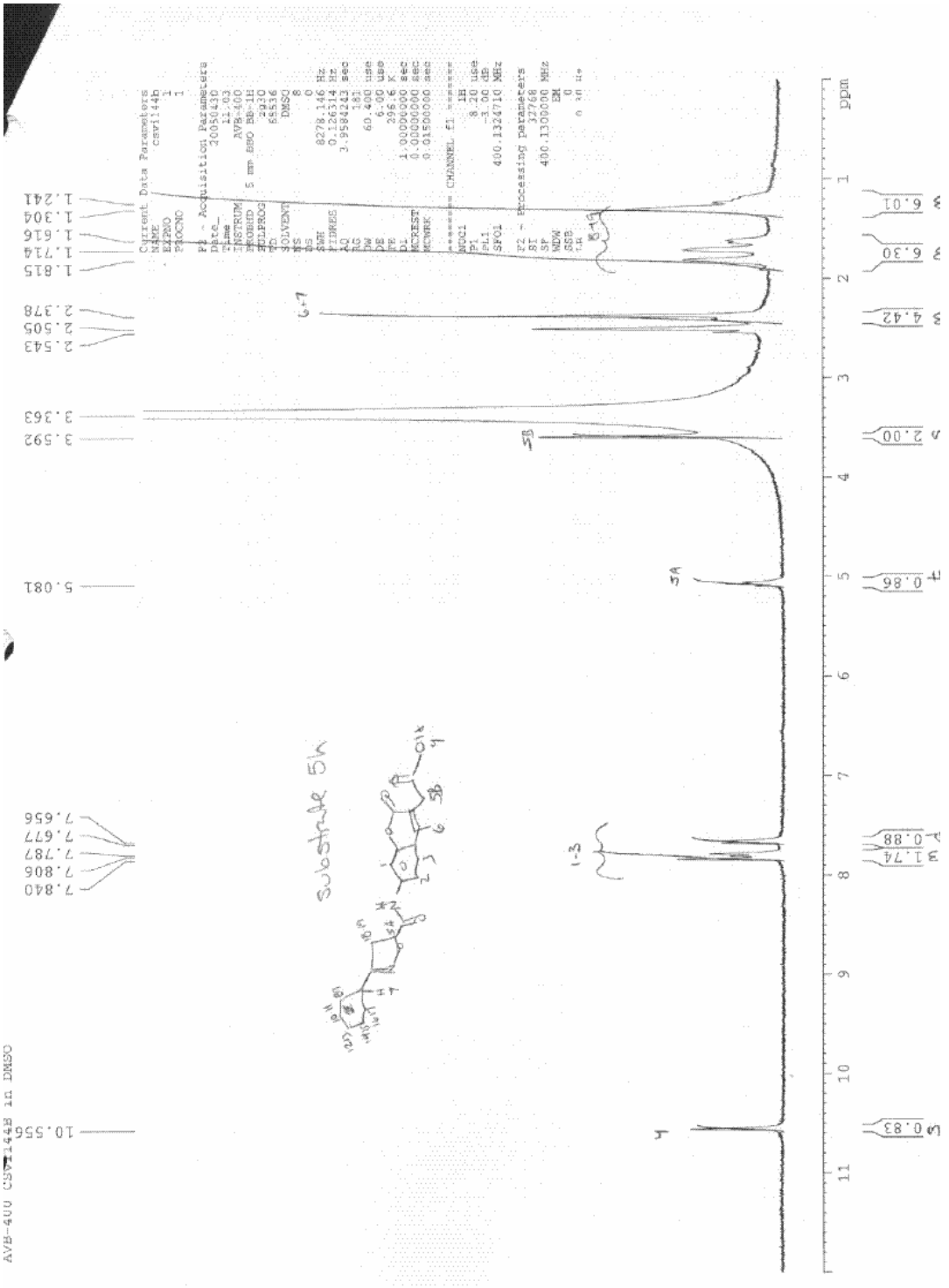
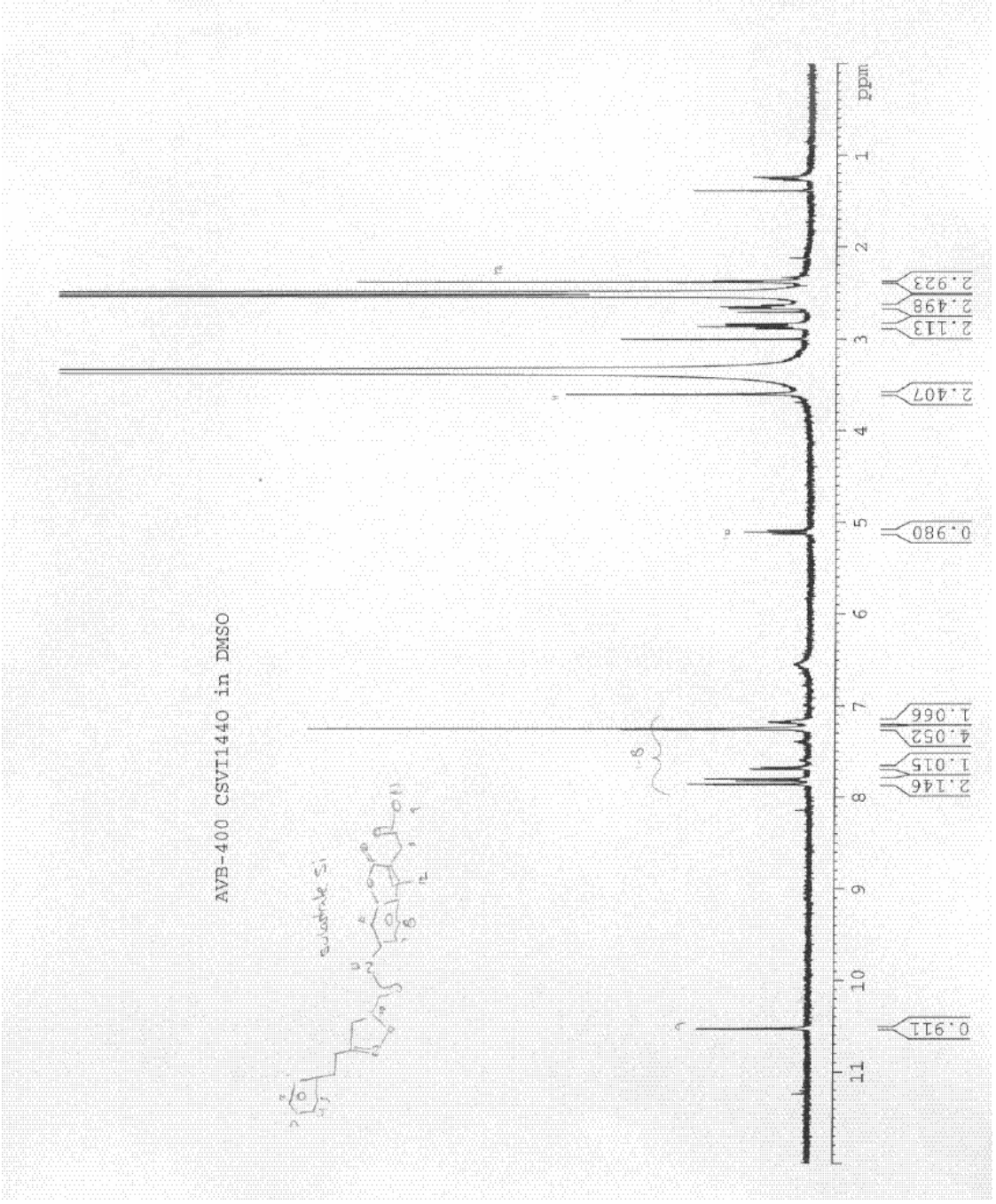
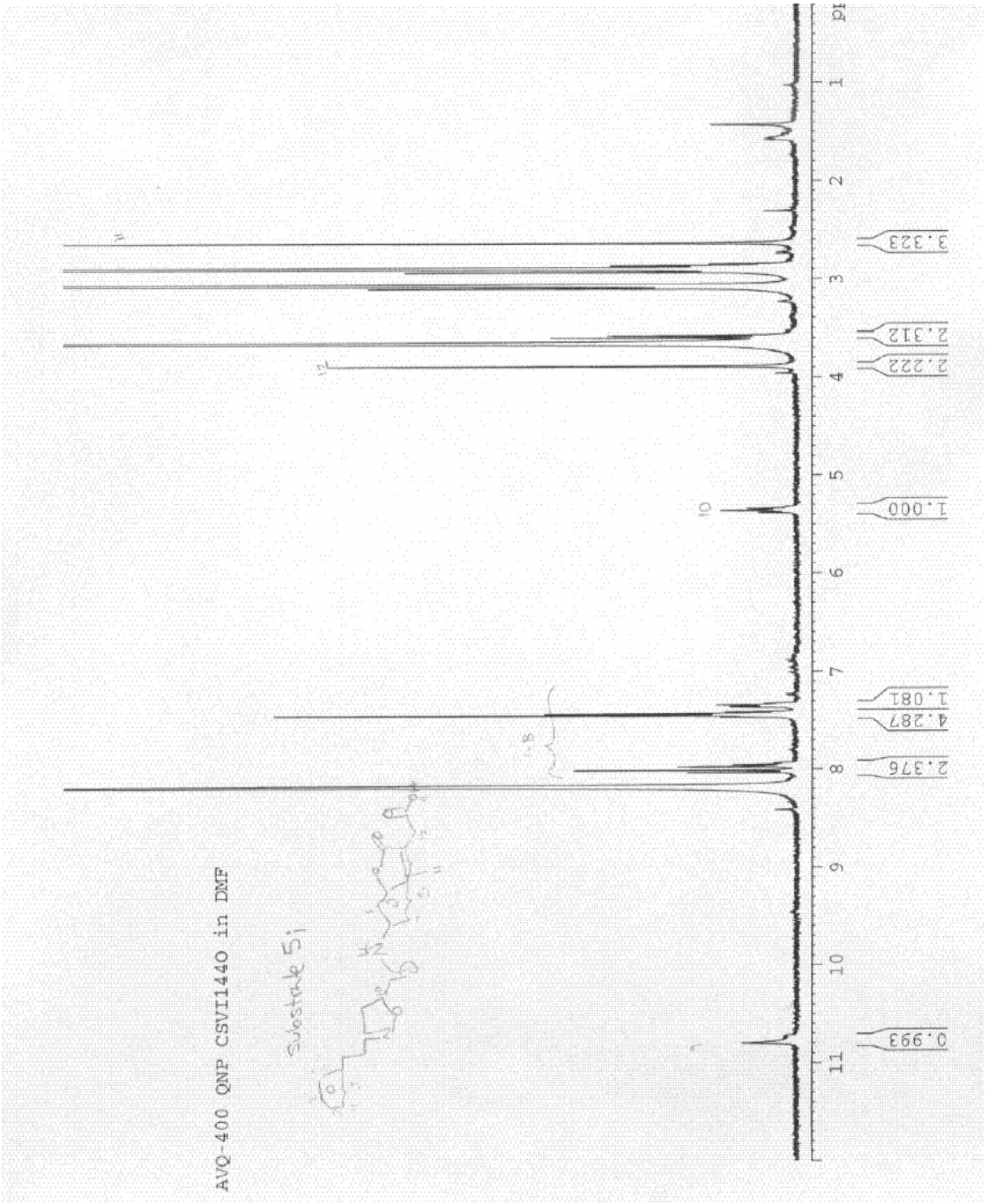


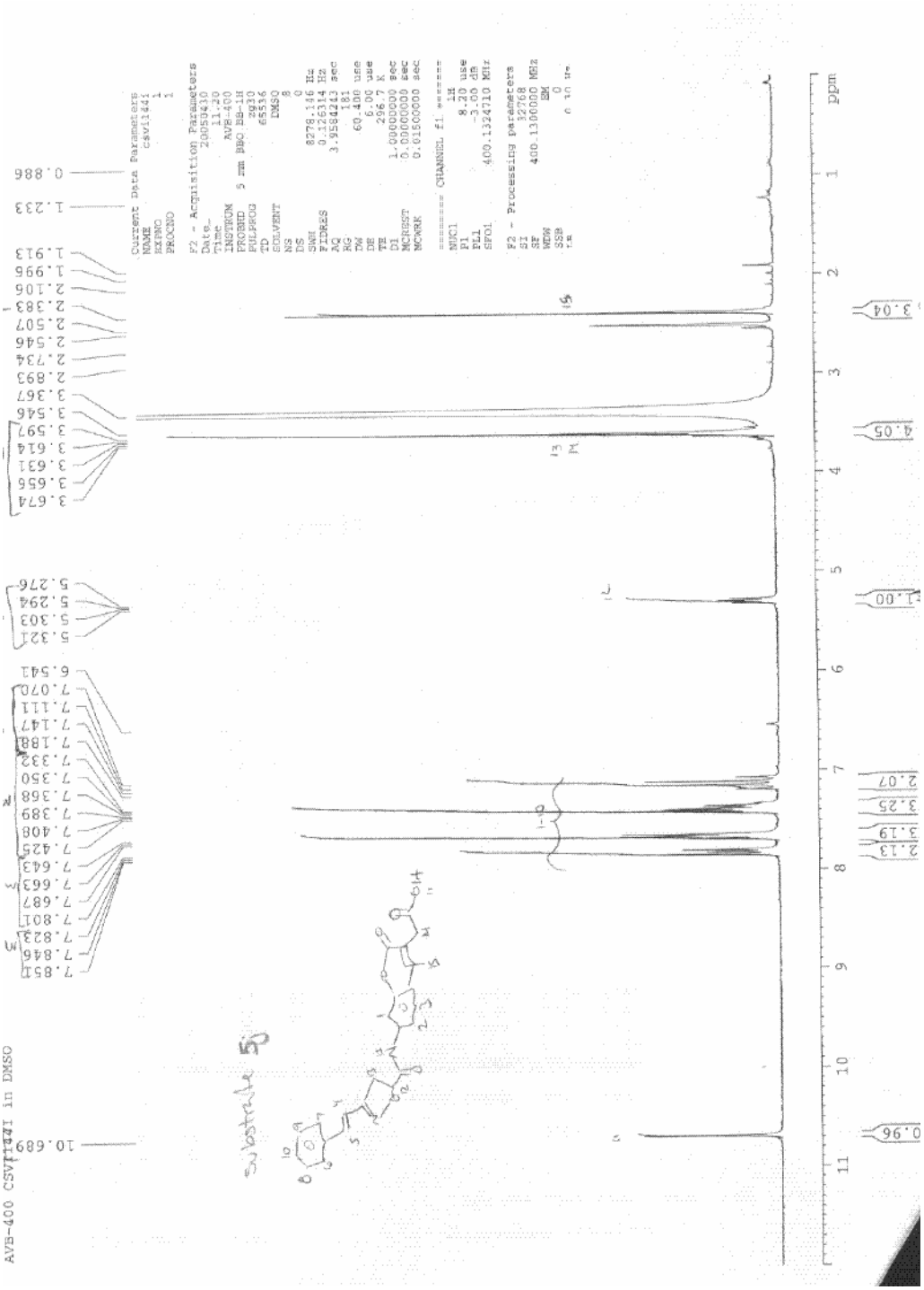
Substrate **5i** in DMSO



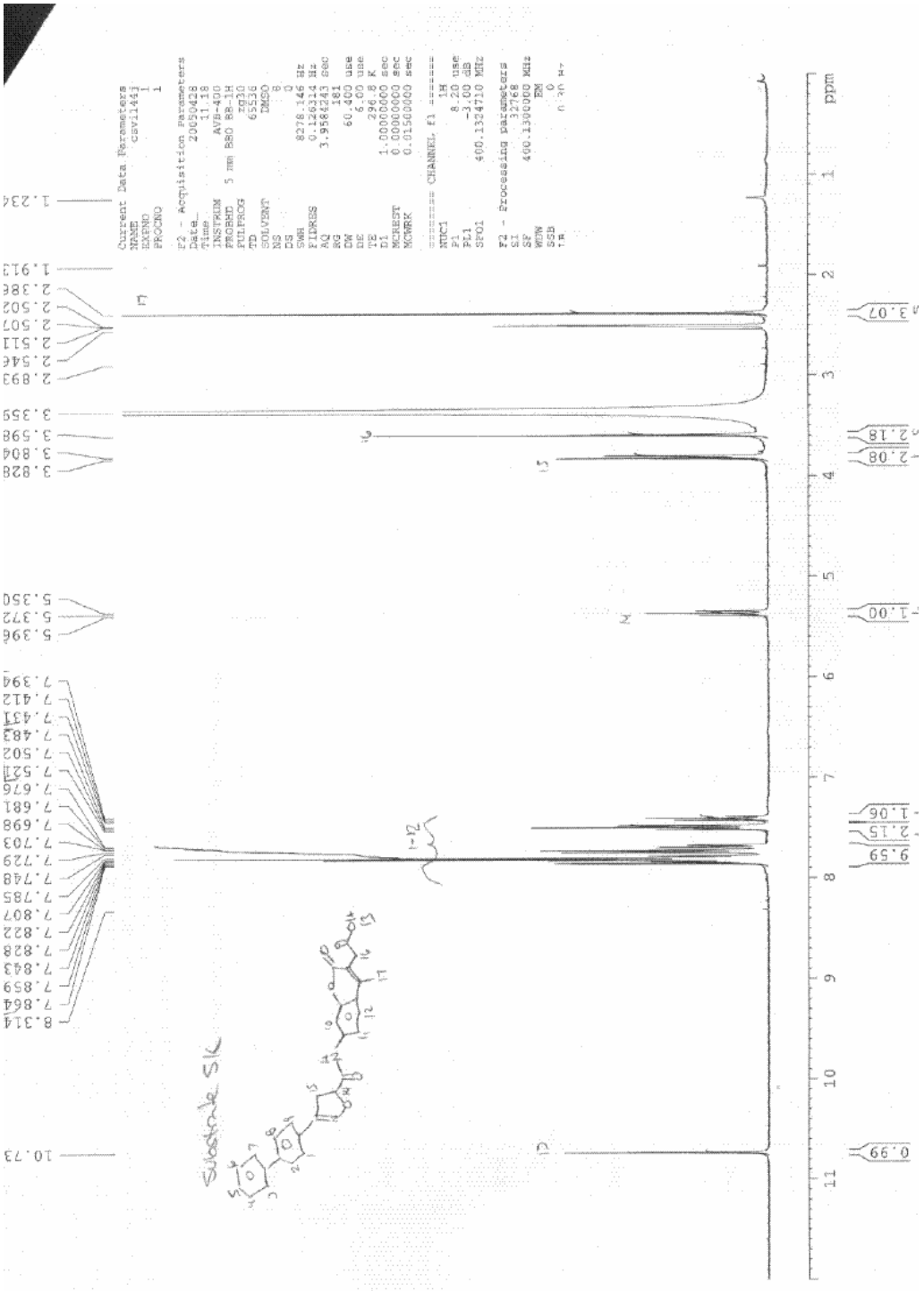
Substrate **5i** in DMF



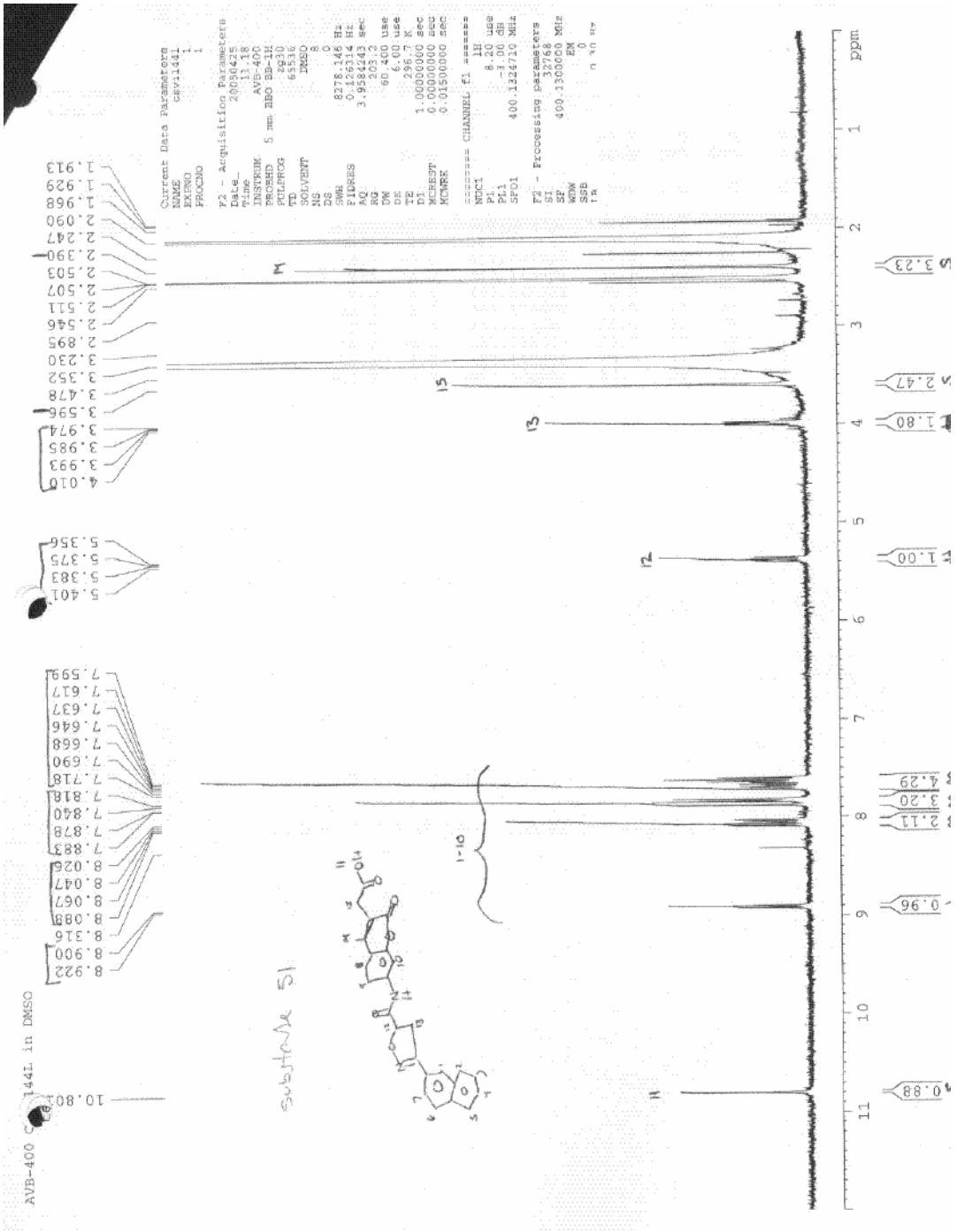
Substrate **5j**



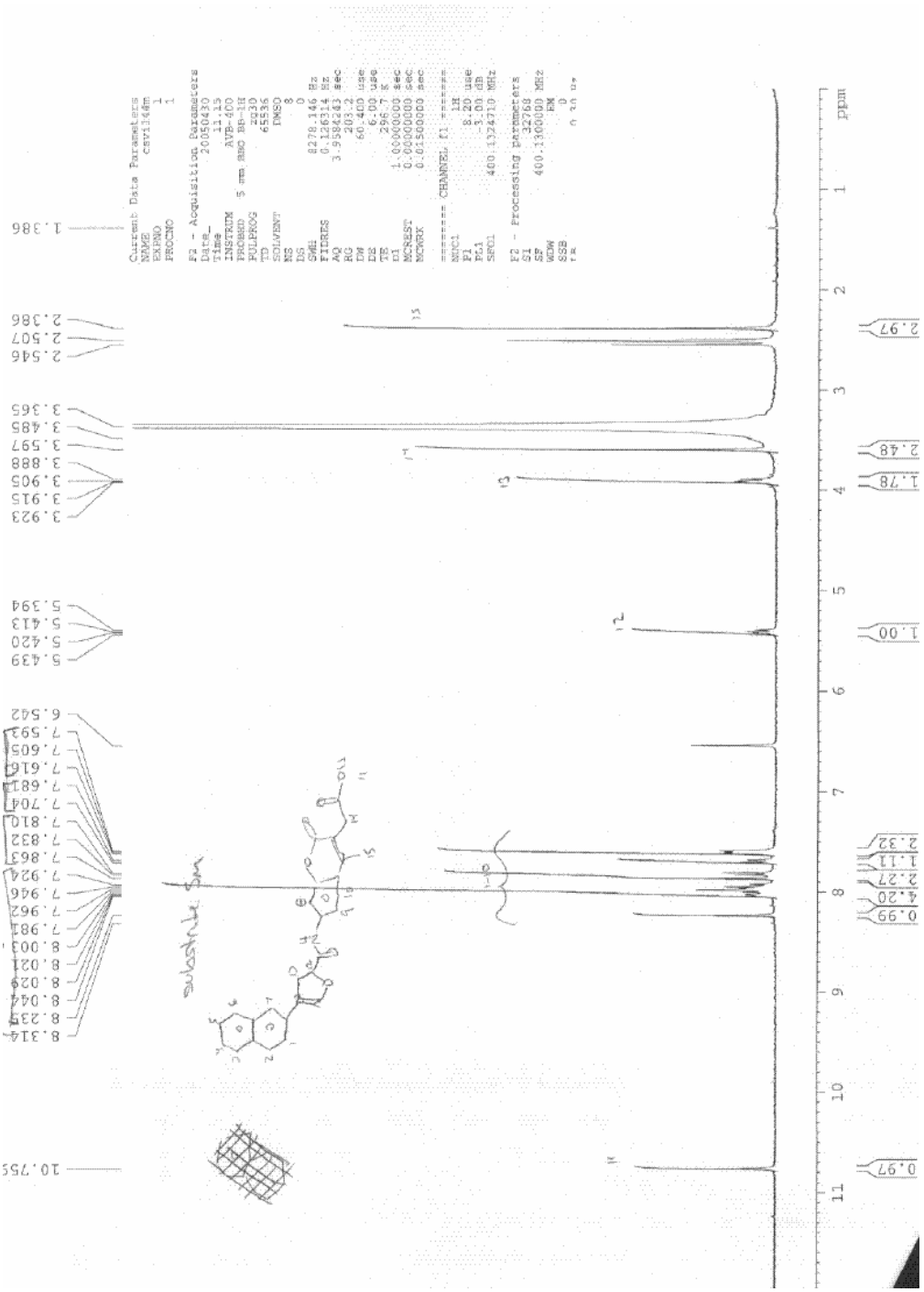
Substrate **5k**



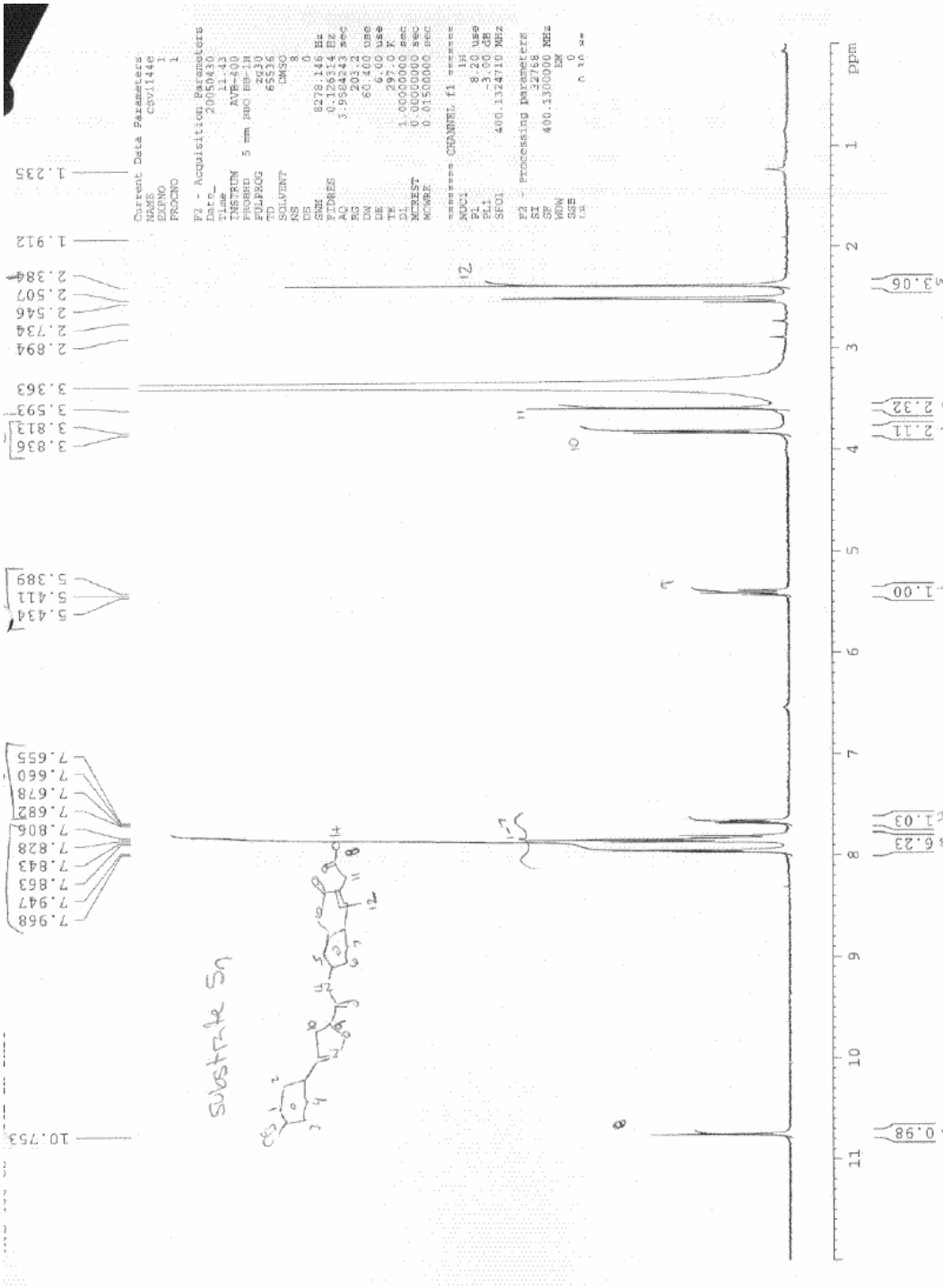
Substrate **5l**



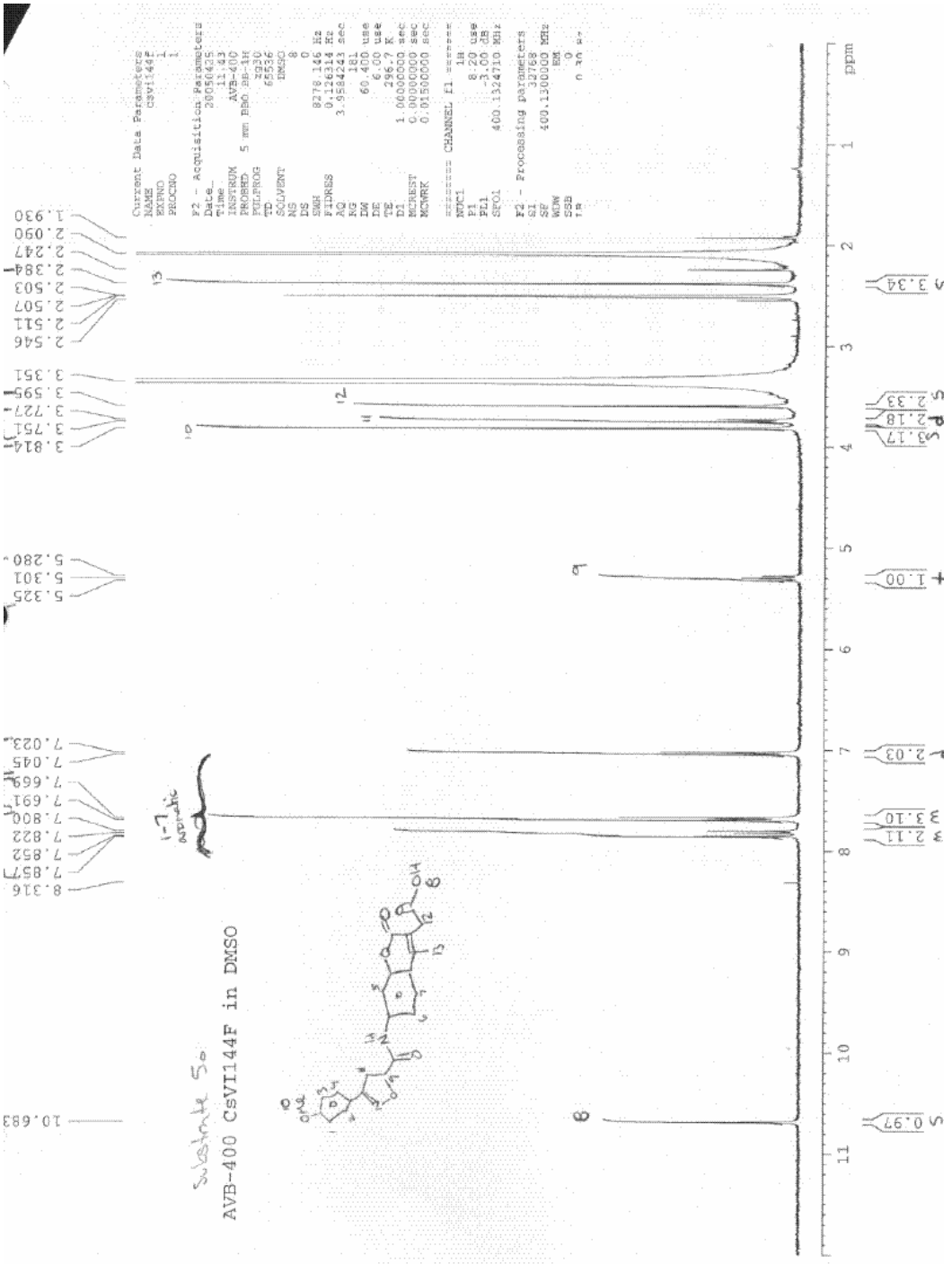
Substrate **5m**



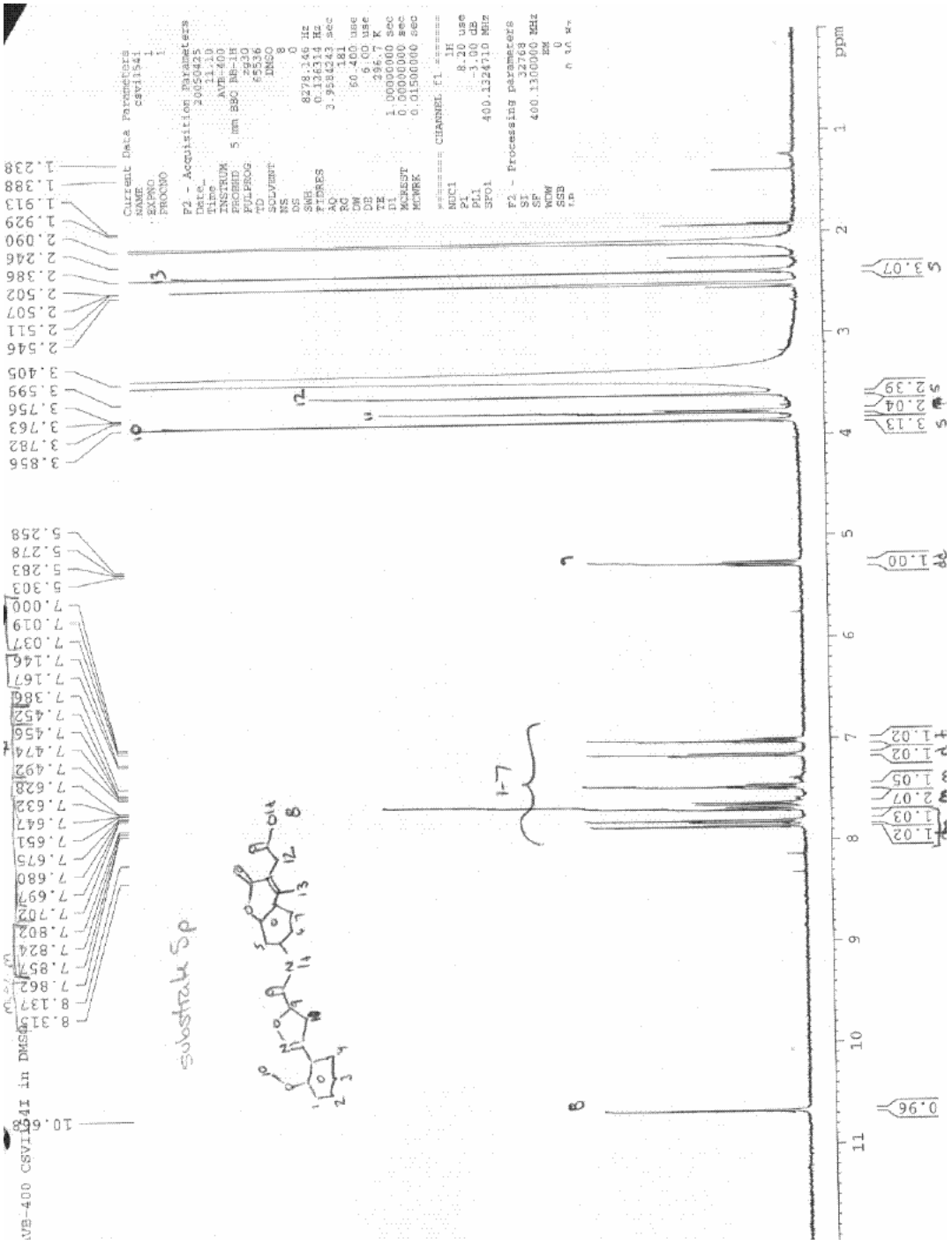
Substrate **5n**



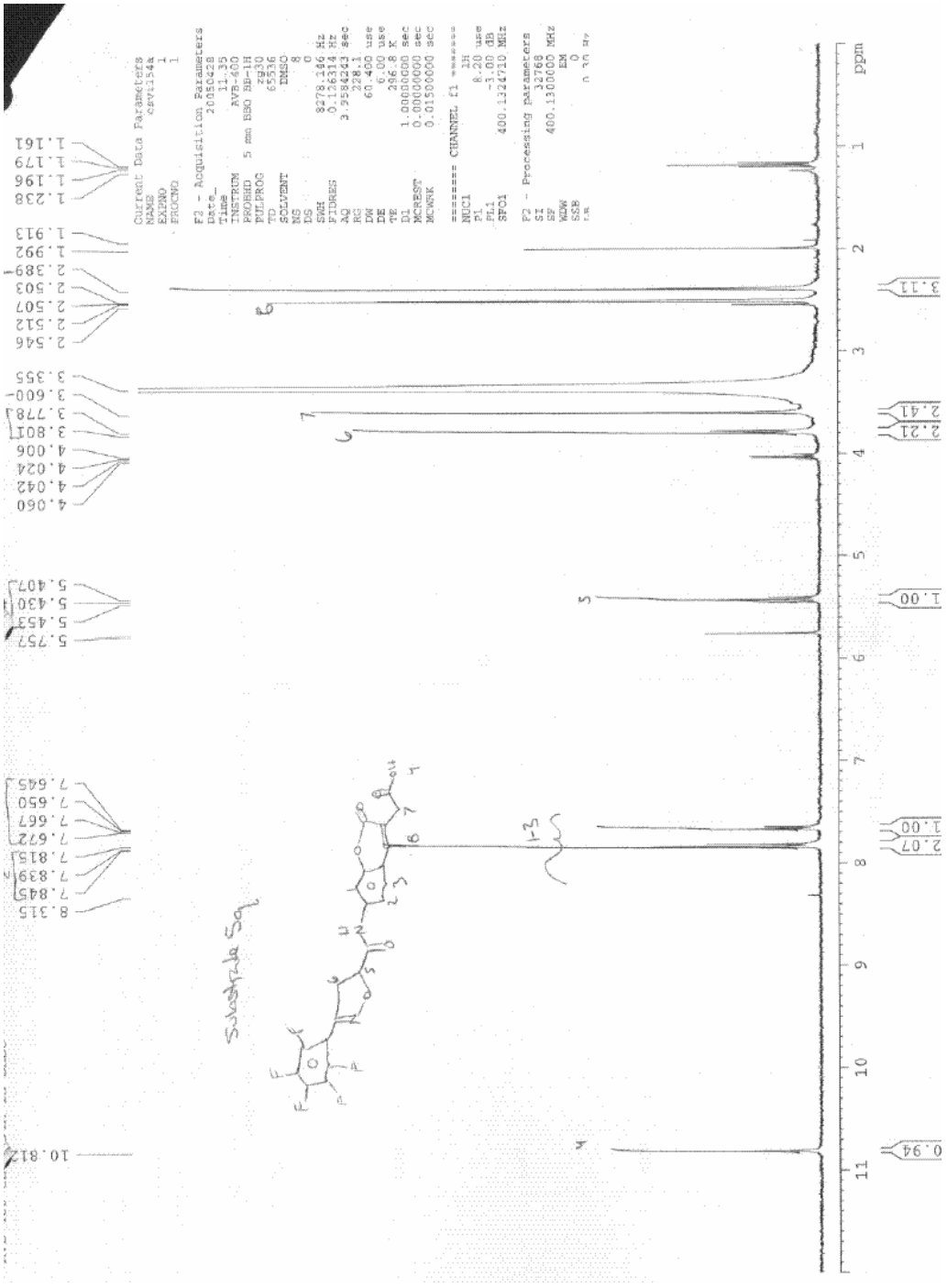
Substrate **5o**



Substrate **5p**

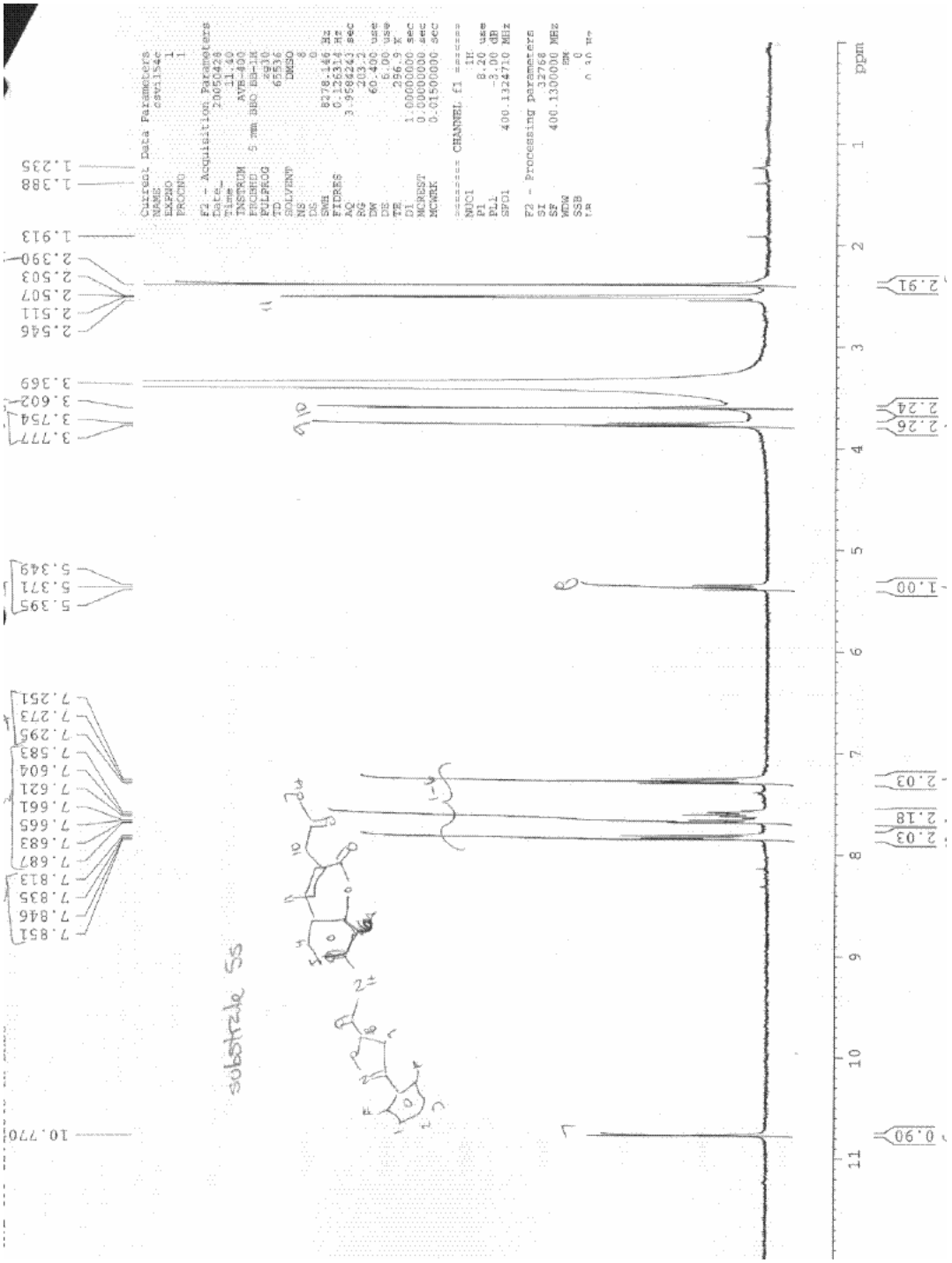


Substrate **5q**

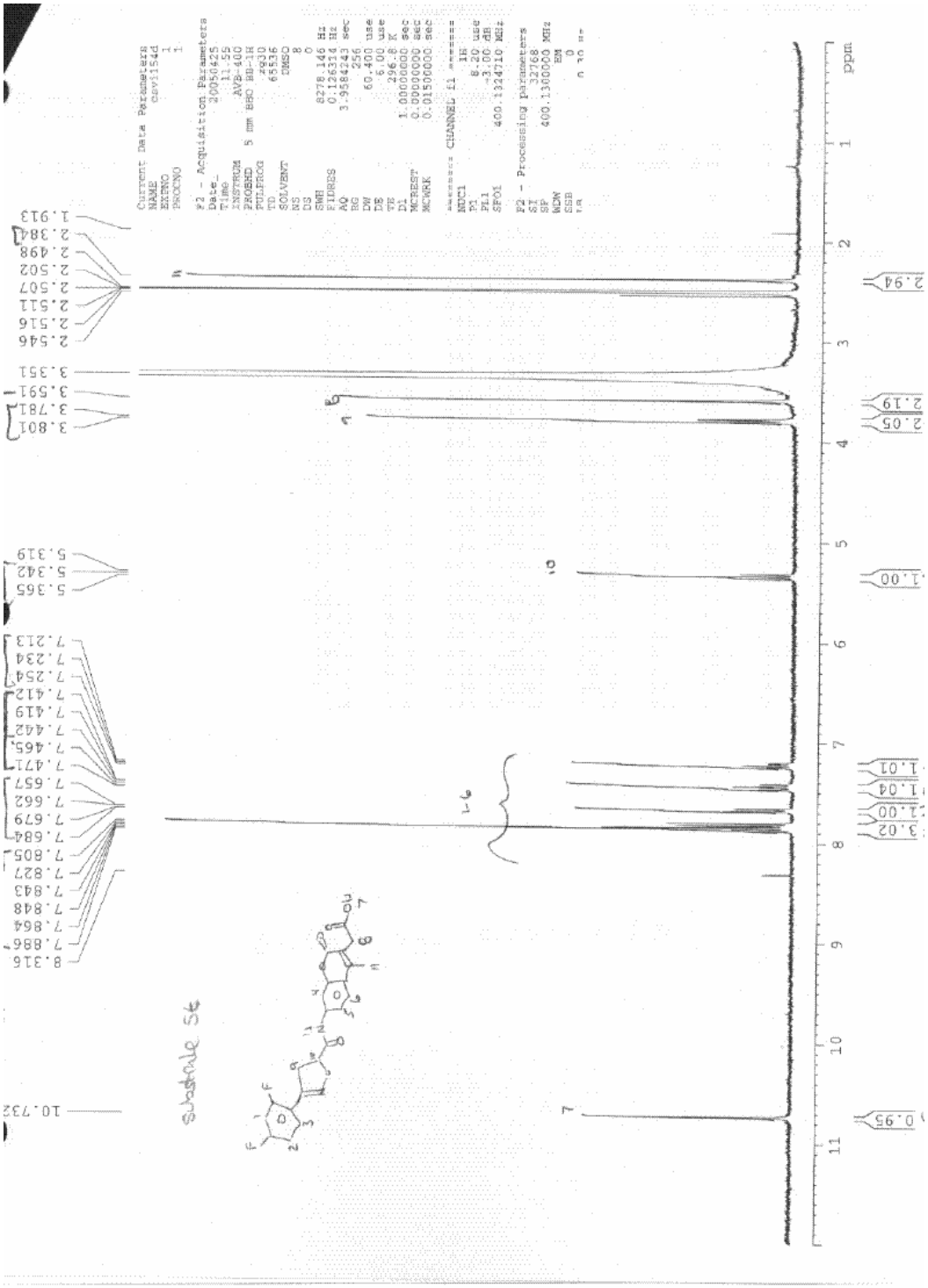


Substrate **5r**

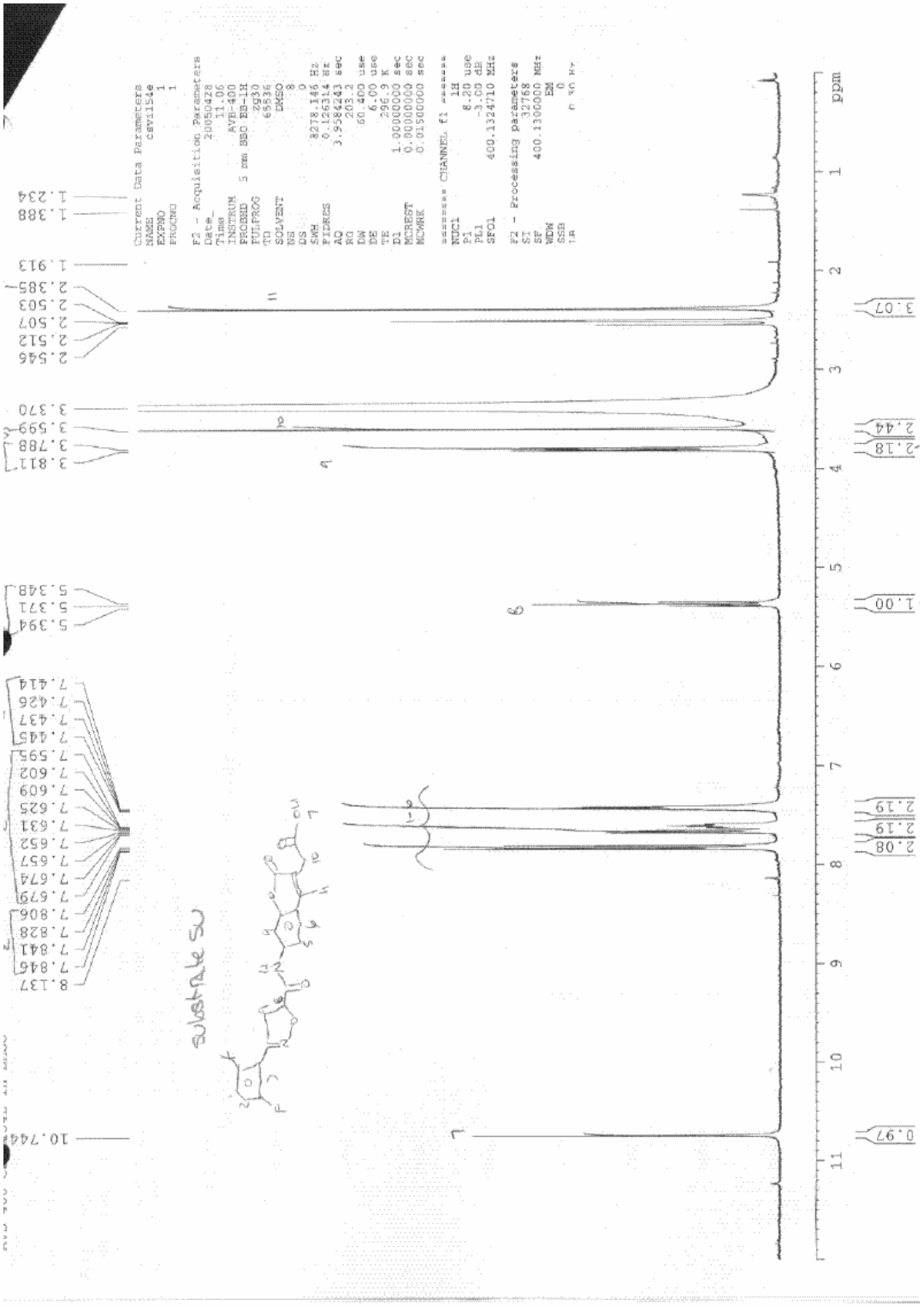
Substrate **5s**



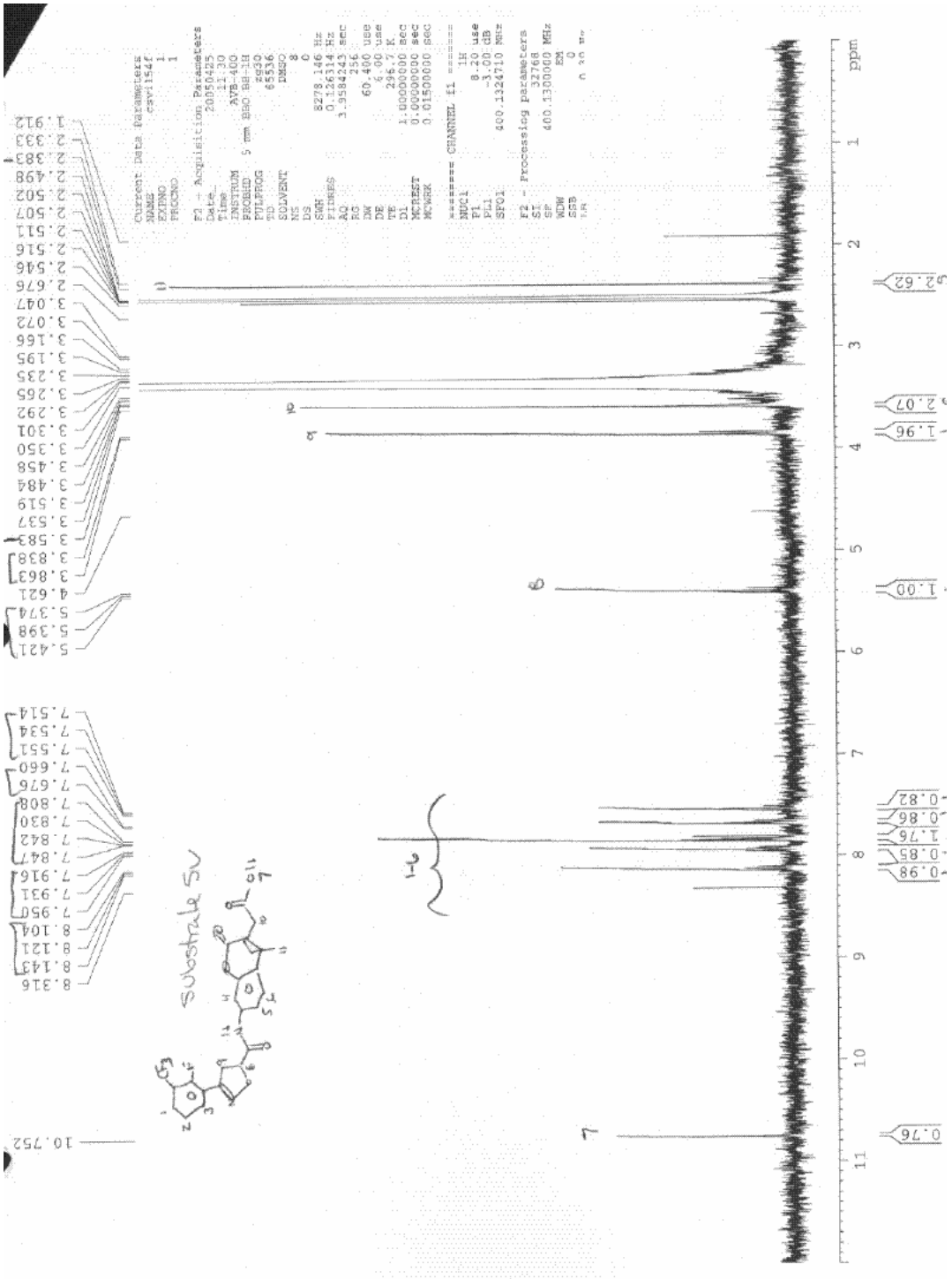
Substrate **5t**



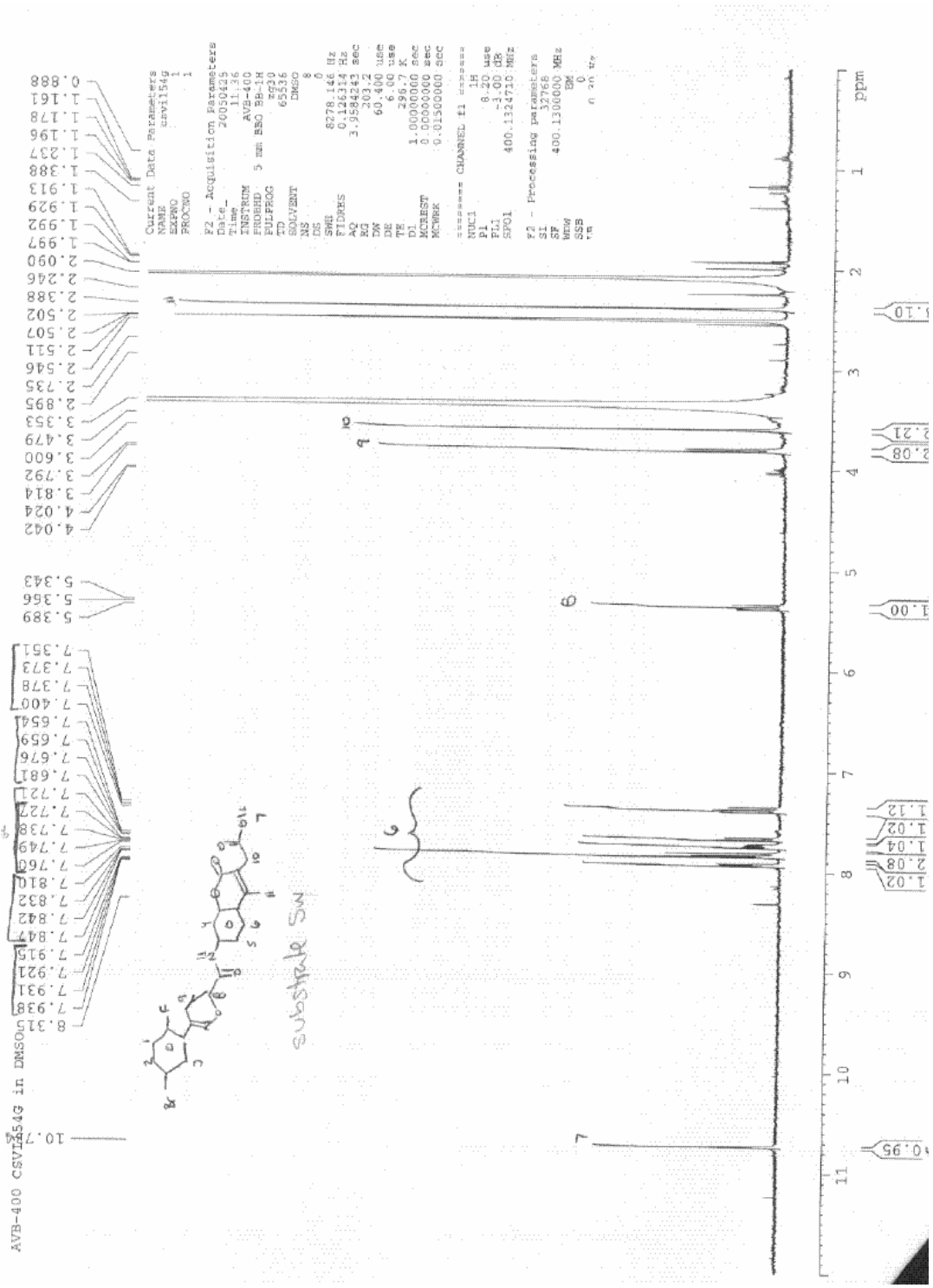
Substrate **5u**



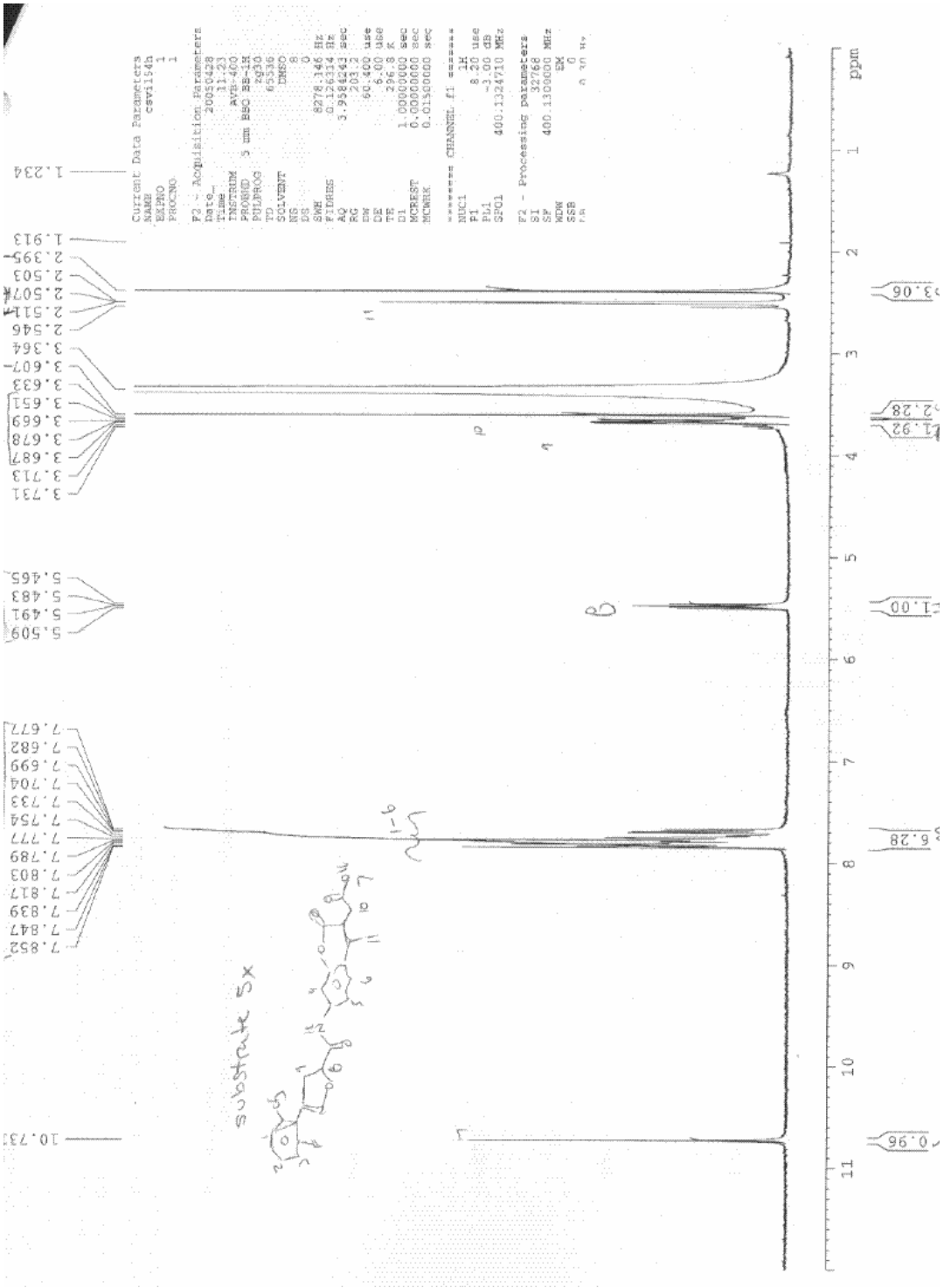
Substrate **5v**



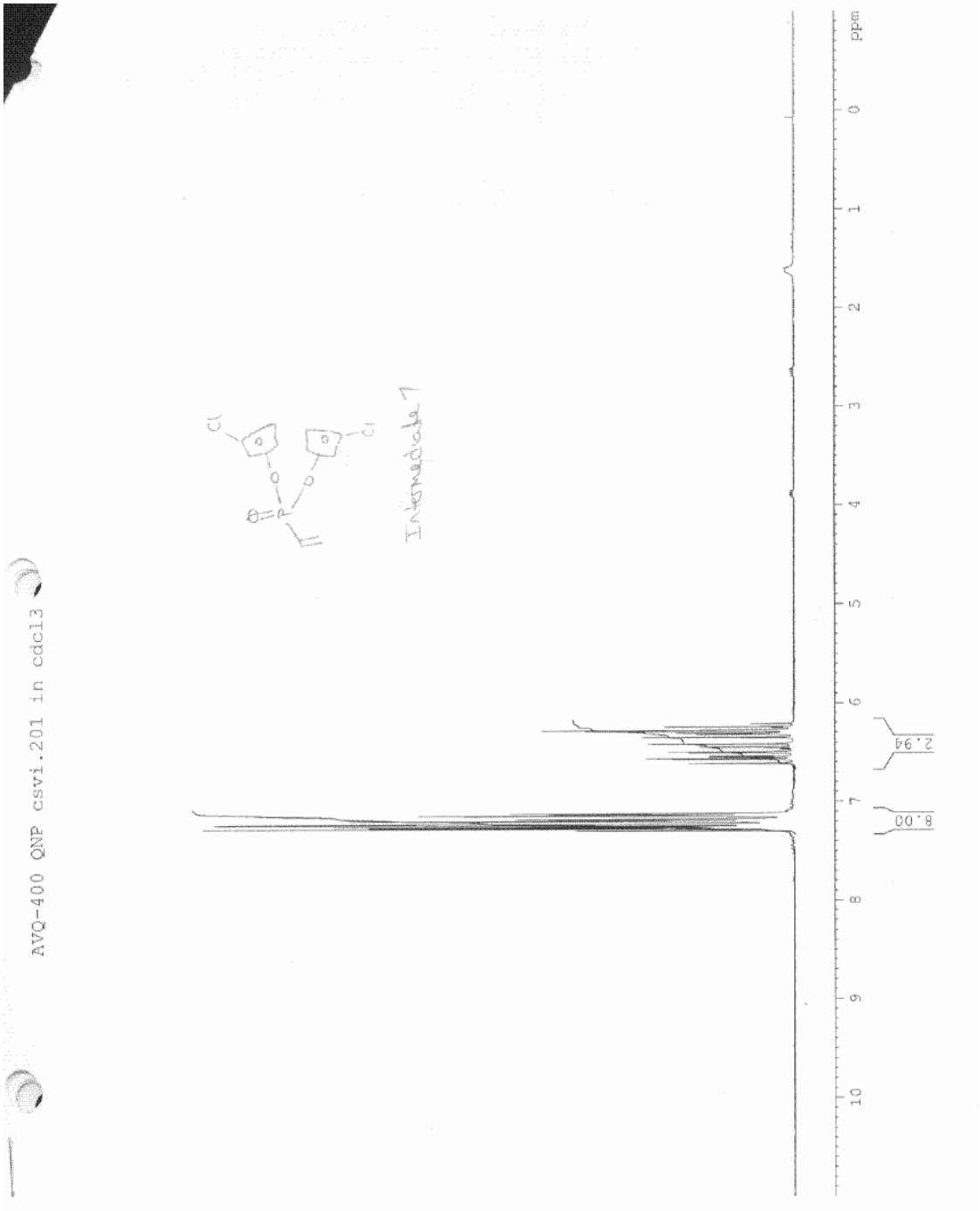
Substrate **5w**



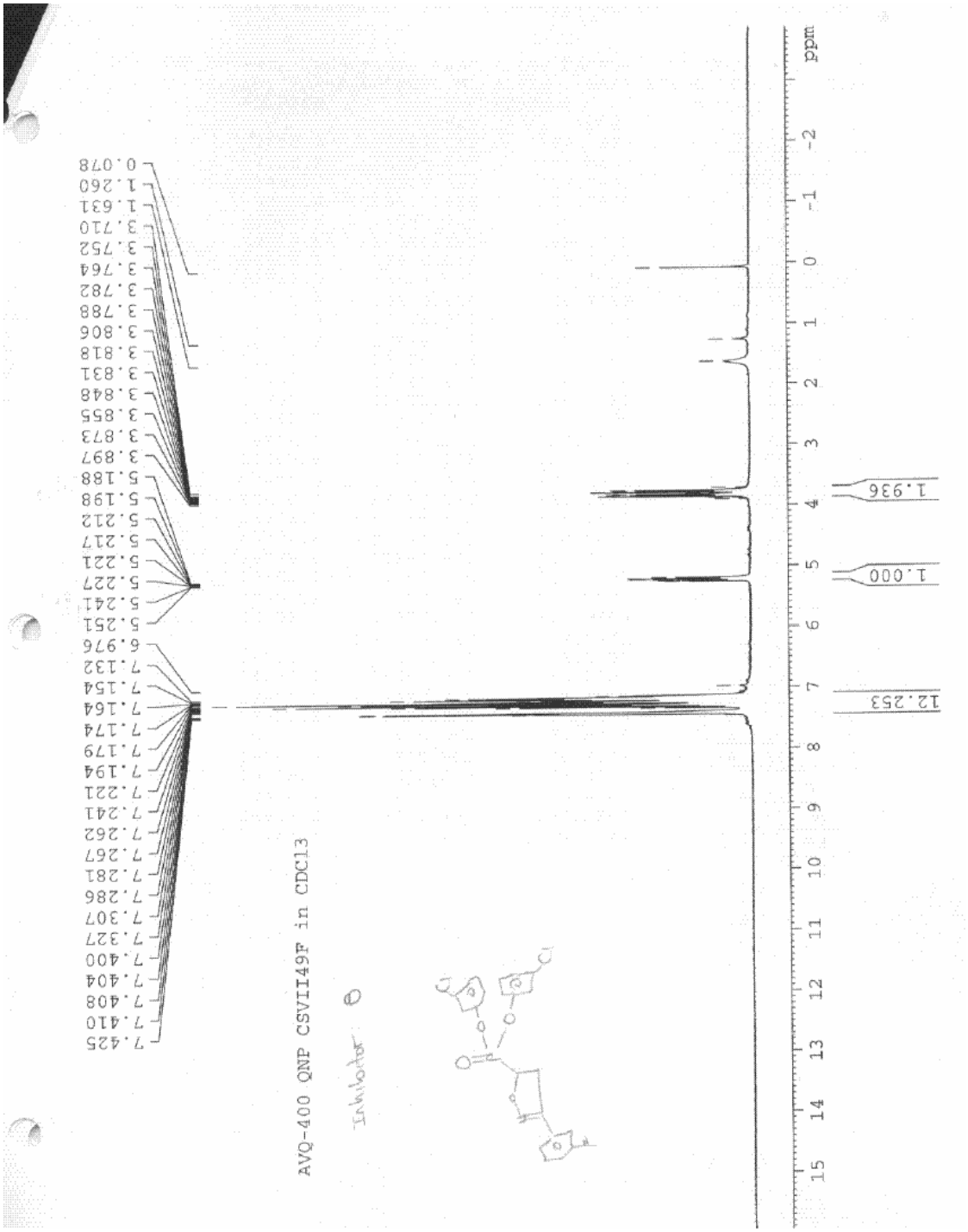
Substrate **5x**



Intermediate **7**



Inhibitor **8**


AVQ-400 QNP CSVII49F in CDCl3
Inhibitor 8
ppm
12.253
1.000
1.936

Minimal Warhead **9**