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Probing the Human Estrogen Receptor- α Binding Requirements for Phenolic Mono- and Di-Hydroxyl Compounds: A Combined Synthesis, Binding and Docking Study

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The spectral data used in the research of this article may be found here.

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Probing the Human Estrogen Receptor- α Binding Requirements for Phenolic Mono- and Di-Hydroxyl Compounds: A Combined Synthesis, Binding and Docking Study

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Abstract: Various estrogen analogs were synthesized and tested for binding to human ER α using a fluorescence polarization displacement assay. Binding affinity and orientation were also predicted using docking calculations.

Docking was able to accurately predict relative binding affinity and orientation for estradiol, but only if a tightly bound water molecule bridging Arg394/Glu353 is present. Di-hydroxyl compounds sometimes bind in two orientations, which are flipped in terms of relative positioning of their hydroxyl groups. Di-hydroxyl compounds were predicted to bind with their aliphatic hydroxyl group interacting with His524 in ER α . One nonsteroid-based dihydroxyl compound was 1000-fold specific for ER β over ER α , and was also 25-fold specific for agonist ER β versus antagonist activity. Docking predictions suggest this specificity may be due to interaction of the aliphatic hydroxyl with His475 in the agonist form of ER β , versus with Thr299 in the antagonist form. But, the presence of this aliphatic hydroxyl is not required in all compounds, since mono-hydroxyl (phenolic) compounds bind ER α with high affinity, via hydroxyl hydrogen bonding interactions with the ER α Arg394/Glu353/water triad, and van der Waals interactions with the rest of the molecule.

Keywords: Estrogen receptor, Docking, Phenolic, Breast cancer, Endocrine disruptor

1. Introduction

Estrogen receptor- α (ER α) is a 595-residue, 66 kDa protein with a ligand binding domain of 245 residues (28 kDa). ER α , along with estrogen receptor- β (ER β), belongs to the nuclear hormone family of intracellular receptors. It is one of the two principal receptors responsible for binding the endogenous estrogen, 17 β -estradiol (E2), shown in Figure 1.¹ In the nucleus, ER binds to DNA as a dimer, recruiting coactivators or corepressors that will result in activating or repressing the transcription of different genes.³ Binding of E2 activates the ER, regulating activity. Both ER α and ER β forms are found in different tissue types. However, ER α is expressed more in breast tissue and is also known to be involved in the pathway that regulates breast cancer development.^{2,4} ER α antagonists such as raloxifene (Fig. 1) can bind to ER in the same ligand-binding domain as E2, and disrupt normal ER cellular function.^{4,5}

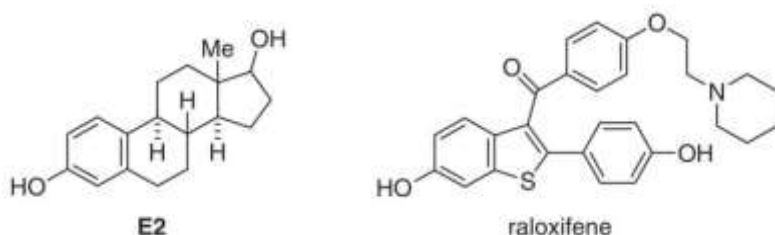


Figure 1 Structures of 17 β -estradiol and raloxifene.

A key structural feature of E2 is the presence of two hydroxyl groups that are separated by 11 Å, which permits interaction with conserved binding site residues Arg394/Glu353 and His 524. But, the receptor is capable of binding many other compounds whose structures resemble that of the E2 hormone.⁶ Some of these compounds are endogeneous, such as estrone and other human estrogens; and, some are exogeneous, like the drugs raloxifene (Fig. 1) or tamoxifen that are used to treat breast cancer and osteoporosis.⁷ In addition to drugs, there exist other exogeneous compounds, some naturally occurring like phytoestrogens and some synthetic such as organochlorines, that have measurable estrogenic activity.⁵ Many of these latter compounds have been shown to be linked to breast cancer as well as birth defects.^{8,9} Through the National Institutes of Environmental Health Sciences, the BSB (Biomolecular Screening Branch), and other federal agencies, the government has developed a program to test many of the chemicals currently in our environment, to see if they have estrogenic activity.¹⁰

Because of the estrogen receptor's prominent role as a breast cancer drug target, along with the threat posed by the potentially large number of estrogen agonists and antagonists in our environment (e.g., endocrine disruptors), it is essential to gain a better understanding of the binding requirements of the ER α ligand pocket. This understanding will allow for the design of better breast cancer drugs that interfere with the carcinogenic activity of estrogen agonists, and improve our ability to predict which pollutants might bind to ER α . Such predictions are strengthened by a better definition of the molecular features that trigger agonist or antagonist effects, as well as a validation of the docking methods used to predict binding.

One technique that can provide a quick and reliable experimental measurement of binding affinity is fluorescence polarization.¹¹ A fluorescence polarization displacement assay can be used to screen non-fluorescent molecules, by displacing a fluorescent probe with the molecule of interest.¹² Such fluorescence polarization displacement assays have been developed previously for ER α and ER β , based on a fluorescein isothiocyanate (FITC)-tagged estradiol (F-E2).^{13,14} One such assay is available from Invitrogen.¹⁵ Subsequent studies in our lab improved the synthesis of F-E2 and examined the *in vivo* behavior of F-E2 *in vivo*, in fish. F-E2 was found to localize in cells

that develop into reproductive organs, consistent with the proposed role of E2 in gender determination in fish.¹⁶ An analogous fluorescence polarization method was developed using an intrinsically fluorescent nonsteroid estrogen.¹⁷

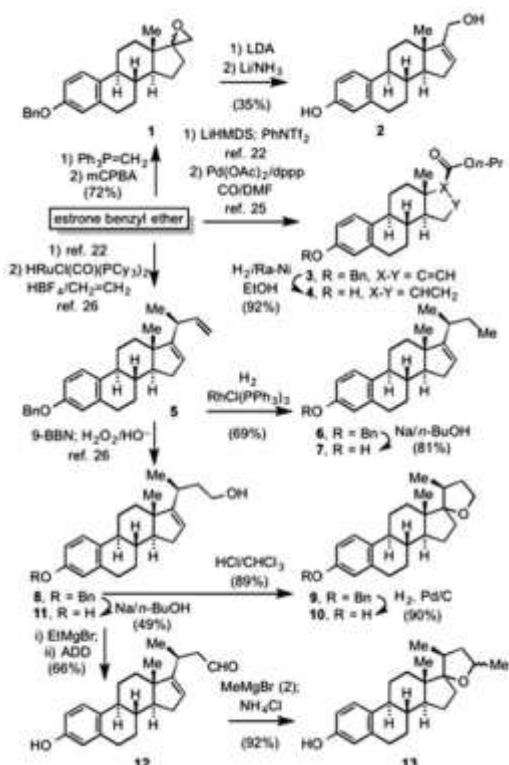
Herein we present the synthesis of a series of phenolic mono- and di-hydroxyl estrogen analogs, which were tested for binding affinity for human ER α , using a fluorescence polarization displacement assay based on F-E2. Estrogen (E2) is a phenolic compound comprised of a steroid core and a second hydroxyl group that is 11 Å from the phenolic hydroxyl. Compounds synthesized herein have the phenolic core, but vary in terms of whether they: (a) are steroid-based, and (b) possess a second hydroxyl group, ~11 Å from the phenol. In addition to binding affinity measurements for compounds, docking calculations were performed. Docking is the process of positioning a ligand into the binding site of a protein and calculating a binding energy for each pose.¹⁸ It has become an important early-stage method for finding molecules likely to bind to a protein, allowing for many chemicals to be rapidly screened as potential drug leads.¹⁸⁻²⁰ Docking has also proven useful for identifying compounds as targets for pollutant remediation.²¹ Besides predicting relative binding affinity, docking is used to predict the orientation or pose of a known ligand bound to a protein.²² Comparison of docking predictions with experimental affinity measurements allows one to rationalize binding site requirements, and also provides validation of the predictive ability of the docking calculations for a given target (e.g., ER α) and class of compounds (phenolic mono- and di-hydroxyl compounds). This is important because such experimental validation provides greater confidence in the docking calculations when they are done on larger sets of compounds, where experimental verification might not be feasible.

2. Results and discussion

2.1. Synthesis

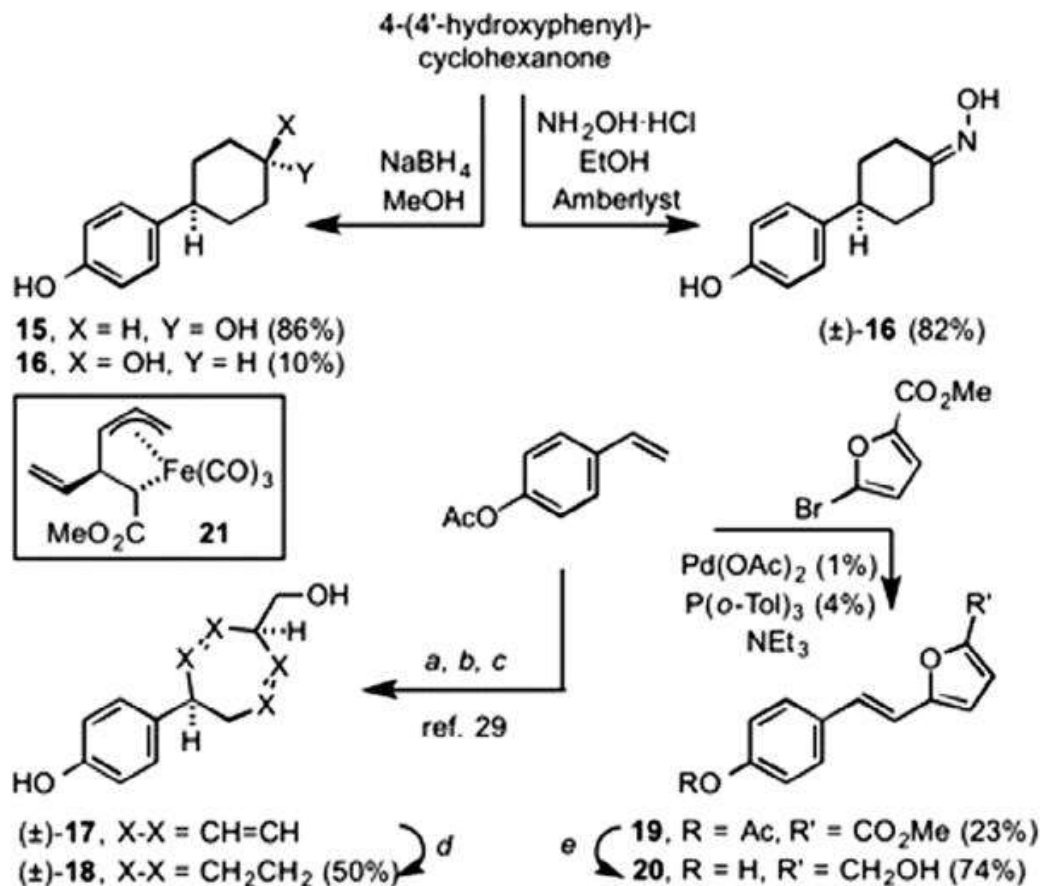
Wittig olefination of estrone benzyl ether,²³ followed by epoxidation with mCPBA gave the known²⁴ epoxide **1** as a mixture of diastereomers (Scheme 1). Deprotonation of **1** with lithium diisopropylamine, followed by cleavage of the benzyl ether under

dissolving metal conditions gave the allylic alcohol **2**. Palladium catalyzed alkoxy carbonylation of the vinyl triflate derived from estrone benzyl ether, according to the literature procedure,²⁵ gave *n*-propyl (20*S*)-3-(phenylmethoxy)-estra-1,3,5(10),16-tetraene-17-carboxylate (**3**), which upon reduction in the presence of Raney-Ni gave the saturated ester **4**. The skipped diene (20*S*)-3-(phenylmethoxy)-19,24-dinorchola-1,3,5(10),16,22-pentaene (**5**) was prepared by the literature procedure.²⁵ Hydrogenation of the less substituted olefin in the presence of Wilkinson's catalyst, followed by debenzoylation gave **7**. Hydroboration-oxidation of **5**, by the literature procedure²⁶ gave (20*S*)-3-(phenylmethoxy)-19,24-dinorchola-1,3,5(10),16-tetraen-23-ol (**8**). Subjecting **8** to acid resulted in the spirocyclic tetrahydrofuran **9** in quantitative yield, which upon catalytic hydrogenolysis gave **10**. Alternatively, debenzoylation of **8** afforded **11**. Oxidation of **11** gave the aldehyde **12**. Reaction of **12** with an excess of methyl Grignard, followed by work-up with saturated aqueous ammonium chloride proceeded by cyclization to afford the spirocyclic tetrahydrofuran **13** as a mixture of diastereomers.



Scheme 1 Preparation of tetra- and pentacyclic ER analogs (ADD = 1,1'- (azodicarbonyl)dipiperidine).

A series of *p*-substituted phenols were also prepared (Scheme 2). Reduction of 4-(4'-hydroxyphenyl)cyclohexanone gave a separable mixture of *trans*-4-(4'-hydroxy-cyclohexyl)phenol **15** (86%) and its *cis*- diastereomer **14** (10%). The stereochemical assignments for each were made by comparison to their literature spectral data.²⁷ Reaction of 4-(4'-hydroxyphenyl)cyclohexanone with hydroxylamine-hydrochloride gave the oxime **16**. [4-((4'-Hydroxyphenyl) cyclohepta-2,6-dienyl)methanol **17** was prepared from *p*-acetoxystyrene according to the literature procedure.²⁸ This involved cross metathesis with (1-methoxycarbonyl-2-vinyl-3-pentene- 1,5-diyl)Fe(CO)₃ (**21**), followed by oxidatively induced reductive elimination. Reduction of the resultant cyclopropane-carboxylate and concomitant Cope [3,3]-rearrangement gave the cycloheptadiene **17**. Catalytic reduction of **17** gave the saturated cycloheptane **18**. Finally, Heck-type coupling of methyl 5-bromo- 2-furanoate with *p*-acetoxystyrene gave the *trans*-styrylfuranoate **19**, which upon reduction with lithium aluminum hydride gave the furfuryl alcohol **20**.



Scheme 2 Preparation of *p*-substituted phenols. Reagents and conditions: (a) **21**, Grubbs 1st generation catalyst; (b) H₂O₂/NaOH; (c) LiAlH₄, then 160 °C; (d) H₂, 20% Pd/C, MeOH; (e) LiAlH₄, Et₂O. (See above-mentioned reference for further information.)

2.2. Fluorescence polarization displacement and cell-based ER α and ER β luminescence activity assays

Twelve compounds from Schemes 1 and 2 were screened using fluorescence polarization, for their ability to bind ER α (Table 1). Only six compounds showed any significant affinity for the receptor at concentrations as high as 1 μ M. These compounds include five of the six steroid-core compounds—**2**, **4**, **7**, **11**, and **13**—and one bicyclic compound—**18**. Of the remaining six compounds which did not bind to ER α , one has the steroid core while the others contain the linked ring cores containing a flanking hydroxyl group—a structure whose hydrophobic interior and hydrophilic exterior resembles that of

estrogen itself. The highest affinity ER α ligand was **2**, with a K_d (32 nM) approaching that of E2 (3 nM). **18** is the only non-steroid core compound with measurable ER α binding affinity, but an accurate K_d could not be obtained (estimated to be >1 μ M).

Table 1 Dissociation constants (K_d) from the fluorescence polarization displacement assay and IC₅₀ data from cell-based ER α and ER β agonist assays and ER β antagonist assays

Compound	ER α K_d (nM)	ER α agonist IC ₅₀ (nM)	ER β agonist IC ₅₀ (nM)	ER β antagonist IC ₅₀ (nM)
E2	3 ¹⁵	1.3 ²⁷	46 pM ²⁷	NA
11	320 \pm 40	NA	108 \pm 67	275 \pm 40
4	320 \pm 40	92 \pm 1	9.8 \pm 2	NA
7	160 \pm 10	NA	88 \pm 9	70 \pm 15
13	160 \pm 10	484 \pm 1	111 \pm 26	NA
2	32 \pm 5	145 \pm 1	6.8 \pm 0.2	NA
18	>1 μ M	NA	5.4 \pm 0.3	137 \pm 100

ER α antagonist behavior was not observed. NA indicates data was not of sufficient quality to measure activity. Assay data for E2 binding to ER α ,¹⁵ and ER α agonist and ER β agonist and antagonist activity in cellular assays,²⁷ were previously reported.

Cell-based ER α and ER β luminescence assays were performed to determine whether the ER α ligands were acting as agonists or antagonists, and whether they had specificity for the α isoform (Table 1, Fig. S1–6). Three compounds, **4**, **13**, and **2**, showed agonist activity in the ER α assay; and, all six compounds showed ER β agonist activity, with **4**, **2**, and **18** being the most potent; **18** is unique in its selectivity for ER β over ER α , and is 25-fold more potent as an agonist, versus antagonist. **11**, **7**, and **18** displayed ER β antagonist activity, with **7** being the most potent.

2.3. Docking

Compounds were computationally docked into human ER α and ER β in agonist and antagonist conformations. Poses for ER α are shown in Fig. S7–8. Initial control docking studies were performed with E2, to validate the docking method by demonstrating an ability to reproduce the known binding mode from the crystal structure. Interestingly, E2

docked with similar predicted affinity in two distinct poses for the ER α agonist conformation (Fig. S9, Table S1), essentially flipping the positioning of the two hydroxyl groups with regard to interactions with Arg394/Glu353 and His524, located on opposite sides of the pocket. The predicted pose with the phenolic hydroxyl near Arg394/Glu353 is referred to as the 'normal' mode, and that with the phenolic hydroxyl near His524 as the 'reversed' mode. But, if docking is performed on receptor that has the tightly bound water present near Arg394/Glu353, then only the expected pose is obtained; and, E2 is the ligand with highest predicted affinity (Table 2), as expected. Thus, all docking was performed with the Arg394/Glu353 water present. This binding mode has been studied previously using molecular dynamics, and illustrates the important role of active site water molecules in ligand binding.³⁰

Table 2 Docking of compounds prepared in Schemes 1 and and22 into the agonist and antagonist conformations of ER α and ER β

Compound	Docking score for ERα agonist (kcal mol⁻¹)	Docking score for ERα antagonist (kcal mol⁻¹)	Docking score for ERβ agonist (kcal mol⁻¹)	Docking score for ERβ antagonist (kcal mol⁻¹)
E2	-10.36	-9.70	-10.11	-9.29
4	-10.29	-10.38	-10.66	-10.13
2	-9.82	-9.86	-10.40	-9.71
11	-9.80	-9.30	-10.18	-10.28
7	-9.74	-9.37	-10.00	-10.36
10	-8.82	-9.21	-6.41	-10.08
13	-8.73	-8.82	-4.82	-9.92
18	-8.22	-7.66	-7.86	-7.48
17	-7.37	-7.10	-6.97	-6.83
16	-7.27	-6.99	-6.92	-6.96
20	-6.93	-7.20	-7.34	-7.11
15	-6.85	-6.38	-6.56	-6.77
14	-6.41	-6.28	-6.43	-6.60

Compounds identified as having ER α affinity in the fluorescence polarization displacement assay are in bold.

Docking results were rank ordered according to the lowest energy pose for binding to the ER α agonist conformation, from the cluster with the highest population (Table 2). Identifying the

compounds with measurable K_d values from the fluorescence polarization displacement assay (shown as bold in Table 2) indicates that the docking procedure using Autodock4 was able to separate the binding ligands from the non-binding ligands. ER is a unique docking target, since the binding site is comprised of a nearly closed hydrophobic pocket, flanked by hydrogen bonding groups that could provide specificity.³¹ Care in analyzing docking results is needed due to the large binding area in which ligands can potentially bind, and symmetry of the pocket. Three examples of reversed binding modes that are likely false are shown in Figure 2.

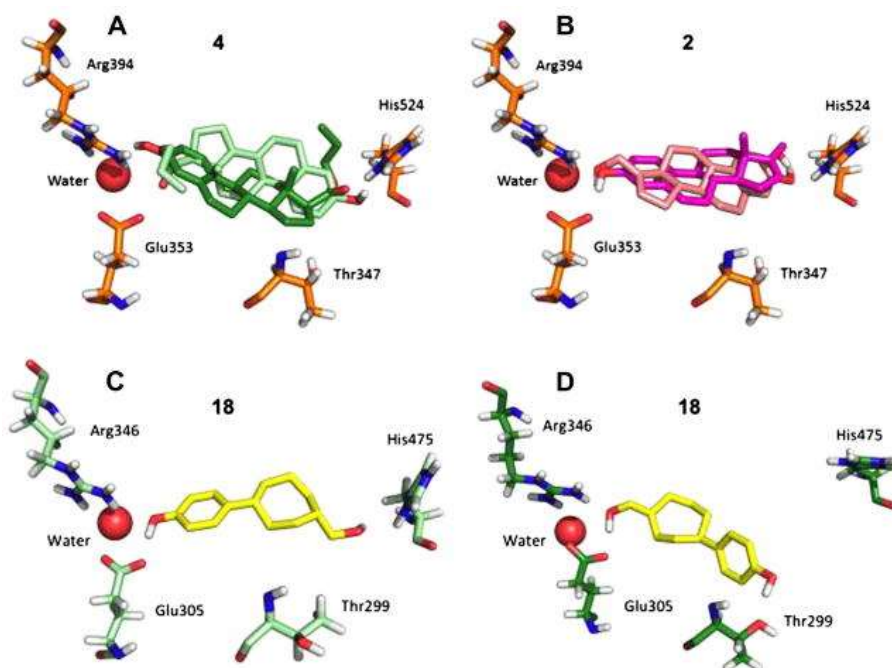


Figure 2 Lowest energy docking poses from clusters where ligands were predicted to bind in two modes (A–B). The human ER α estrogen receptor that was used was in the agonist conformation (PDB code 1ere; chain A). Panel C shows the predicted binding orientation for **18** in ER β , agonist conformation (PDB code 2jj3; chain A). Panel D shows the predicted binding orientation for **18** in ER β , antagonist conformation (PDB code 1l2j; chain A).

Interestingly, while estradiol docked in only one orientation when the bound water is present, other compounds were still predicted to bind in two orientations (Table 2; Fig. 2), one normal (with the phenolic hydroxyl interacting with Arg394/Glu353/Water), and one 'reversed,' where the phenolic hydroxyl interacts with His524. This promiscuity in predicted binding mode may be due to symmetry in di-

hydroxyl molecules like **2** (Fig. 2). Curiously, the mono-hydroxyl **4** also is predicted to bind in a reversed mode (Fig. 2), but with much lower affinity relative to the normal mode. This is likely due to the fact that **4** has only one hydroxyl group, the phenol, which provides significant binding energy via interaction with the Arg394/Glu353/water triad. It is also clear that the aliphatic hydroxyl interaction with His524 is not essential, since it is absent in **4** and **7**, and yet both bind with reasonable affinity ($IC_{50} = 160\text{--}320$ nM). Indeed, this observation is consistent with the ability of phenolic endocrine disruptors, which contain only one hydroxyl group, to bind to ER.³²

The docking of compounds **10** and **13** in the ER β -agonist conformation displayed predicted binding energies that were weaker than expected in Table 2. Inspection of the binding site (Fig. S10) showed that these ligands experience steric clashes with binding site sidechains. Additionally, for structures **10** and **13**, the oxygen atom in the tetrahydrofuran ring was not positioned near His475 for **10** or (for reversed mode binding) near Arg346, Glu305 for **13**, to allow for hydrogen bond formation.

Compound **18** is in a unique class, in that it is not based on the steroid core, is selective for the β over the α ER isoform, and is 25-fold selective for ER β agonist versus ER β antagonist activity (Table 1). Docking pose predictions (Fig. 2C and D) show that **18** could form two hydrogen bonds (one with His475) in the ER β agonist conformation, whereas in the ER β antagonist conformation, hydrogen bonding is with Thr299, rather than His475. A molecular overlay of E2 and **18** (Fig. S11) shows the oxygen atoms of the two molecules are well-aligned.

3. Conclusions

Human ER α remains an important target for therapeutic interventions (cancer; osteoporosis). Estrogen has a key interaction between its phenolic hydroxyl and a binding site Arg394/Glu353/water triad, along with other important interactions including van der Waals interactions with the steroid core, and hydrogen bonding interactions between an aliphatic hydroxyl group and His524 (His475 in ER β). The two estradiol hydroxyls are located 11 Å from each other. The studies presented herein probe the importance of interactions with the

aliphatic hydroxyl and with the steroid core, using a series of novel mono- and di-hydroxyl compounds (Schemes 1 and 2).

The estrogen analog with highest measured affinity in the fluorescence polarization displacement assay ($IC_{50} = 32$ nM) and second highest predicted affinity is the di-hydroxyl steroid **2**, which has a single point of unsaturation in the D-ring, and (relative to estradiol) has its aliphatic hydroxyl extended by one methylene group. Nonetheless, this gives an O–O distance essentially equivalent to that for estradiol. Di-hydroxyl steroid **2** behaves as an ER α agonist, and has no selectivity for α versus β ER isoforms. Indeed, **2** is a potent ER β agonist and antagonist. In contrast, **18** binds weakly to ER α , yet has an O–O distance (11.1 Å) that is similar to **2**. Of particular interest is the fact that **18** has the expected interaction with His475 in the ER β agonist docking, whereas in the ER β antagonist docking this aliphatic hydroxyl group is predicted to interact instead with Thr299 (Fig. 2). This could explain why **18** is so selective (25-fold) as an ER β agonist, versus as an antagonist (Table 1). Most of the other compounds from Scheme 2 that lacked the steroid core did not bind to ER α , even though they possessed the phenolic hydroxyl. Compounds (**4**, **13**, **2**), which possessed ER α agonist activities, were also ER β agonists; but, not ER β antagonists. And, these compounds were more selective for ER β over ER α .

In summary, several compounds have been identified that are potent ER α agonists, and also behave as ER β agonists and antagonists (Table 1). The most potent is the dihydroxyl steroid **2**. Also, the non-steroid dihydroxyl compound **18** is 1000-fold more selective for ER β over ER α , and appears to adopt a different binding mode in these two targets (Fig. 2).

4. Experimental

4.1. General methods

The β -estradiol (min 98%) and fluorescein (FITC) were purchased from Sigma. The α -ER and β -ER screening buffer were from Invitrogen. The FITC-estradiol linked tracer used in the experiments was synthesized by as described previously. (1) DMSO- d_6 was

purchased from Cambridge Isotopes. The 96-well plates used were black, polystyrene, NBS (non-binding surface), flat-bottom plates obtained from Corning. A PolarStar Galaxy fluorescent plate reader was used and controlled with FLUOStar Galaxy software (version 4.30-0). Estrone benzyl ether²³ and compounds **3**,²⁵ **5**,²⁶ **8**,²⁶ and **17**²⁸ were prepared by the literature procedures.

4.2. Estrogen analog synthesis

4.2.1. 3-Hydroxyestra-1,3,5(10),16-tetraene-17-methanol (2)

To a solution of methyl triphenylphosphonium bromide (589 mg, 1.65 mmol) in THF (10 mL) at $-40\text{ }^{\circ}\text{C}$ under N_2 , was added a solution of *n*-butyl lithium (0.66 mL, 2.5 M in hexanes, 1.7 mmol). The ylide solution was warmed to room temperature and a solution of estrone benzyl ether (200 mg, 0.556 mmol) in THF (7 mL) was added. The mixture was stirred for 12 h, and then heated at reflux for 5 h. The solution was cooled, and concentrated, and the residue was purified by column chromatography (SiO_2 , hexanes–ethyl acetate = 4:1) to afford the exocyclic methylene product (168 mg, 84%) as a colorless solid. This product was used in the next step without further characterization. To a solution of the olefin (100 mg, 0.279 mmol) in dichloromethane (6 mL) at $0\text{ }^{\circ}\text{C}$, was added solid *m*-chloroperoxybenzoic acid (57.5 mg, 0.333 mmol). The reaction mixture was 4 h, and then quenched with aqueous NaHCO_3 . The mixture was extracted several times with dichloromethane, dried and concentrated to afford the epoxide **1** (90 mg, 86%) as a colorless oil, which was used in the next step without further purification. To a solution of the epoxide (50 mg, 0.13 mmol) in hexanes (1 mL) and toluene (0.5 mL) was added HMPA (1 drop). The mixture was cooled to $-78\text{ }^{\circ}\text{C}$, and then a solution of lithium diisopropylamine in hexanes (0.73 mmol) was added. The solution was warmed to room temperature and stirred for 10 h. The mixture was quenched with saturated aqueous NH_4Cl , and the mixture extracted several times with ether. The combined extracts were dried (MgSO_4) and concentrated, and the residue was purified by column chromatography (SiO_2 , hexanes–ethyl acetate = 3:2) to afford a colorless oil (29 mg, 58%) which was used without further characterization. To liquid ammonia (ca. 10 mL), at $-78\text{ }^{\circ}\text{C}$ was added lithium metal (24 mg, 3.5 mmol),

followed by *t*-butyl alcohol (0.05 mL). To this solution was added a solution of the allylic alcohol (20 mg, 0.053 mmol) in THF (1 mL). The reaction mixture was stirred at $-78\text{ }^{\circ}\text{C}$ for 15 min, and then quenched with NH_4Cl , and diluted with ether. The mixture was warmed to room temperature, and water (10 mL) was added. The mixture was extracted several times with ether followed by extraction with dichloromethane. The combined extracts were dried (MgSO_4), concentrated and the residue was purified by column chromatography (SiO_2 , hexanes–ethyl acetate = 3:2) to afford **2** (9.0 mg, 60%) as a colorless solid. Mp $192\text{--}194\text{ }^{\circ}\text{C}$; ^1H NMR (CDCl_3 , 400 MHz) δ 7.15 (d, $J = 8.4\text{ Hz}$, 1H), 6.64 (dd, $J = 2.8, 8.4\text{ Hz}$, 1H), 6.58 (d, $J = 2.8\text{ Hz}$, 1H), 5.65 (dd, $J = 1.2, 2.8\text{ Hz}$, 1H), 4.80 (br s, OH), 4.32–4.25 (m, 2H), 2.95–2.80 (m, 2H), 2.40–1.70 (m, 11 H), 0.87 (s, 3H); ^{13}C NMR (CDCl_3 , 100 MHz) δ 155.2, 153.5, 138.5, 133.1, 126.4, 124.3, 126.4, 124.3, 115.5, 112.8, 60.4, 56.8, 46.4, 44.6, 37.4, 34.8, 31.1, 29.7, 27.9, 26.6, 16.5.

4.2.2. *n*-Propyl 3-hydroxyestra-1,3,5(10)-triene-17-carboxylate (**4**)

To a solution of **3** (177 mg, 0.411 mmol) in ethanol (10 mL) was added an aqueous slurry of Raney-Ni (60%, 0.6 mL). The reaction mixture was stirred under a H_2 gas (balloon pressure) for 24 h, after which the mixture was filtered through a bed of filter-aid. The filter bed was washed several times with ethyl acetate, and the filtrate was concentrated under reduced pressure to afford **4** as a colorless solid (129 mg, 92%): mp $151.5\text{--}153\text{ }^{\circ}\text{C}$, $[\alpha]_{20\text{D}} +69.5$ (c 0.388, CHCl_3); ^1H NMR (CDCl_3 , 300 MHz) δ 7.17 (d, $J = 8.4\text{ Hz}$, 1H), 6.64 (dd, $J = 2.8, 8.5\text{ Hz}$, 1H), 6.57 (d, $J = 2.7\text{ Hz}$, 1H), 4.55 (br s, OH), 4.10 (dt, $J = 10.8, 6.7\text{ Hz}$, 1H), 4.02 (dt, $J = 10.8, 6.7\text{ Hz}$, 1H), 2.90–2.80 (m, 2H), 2.44 (t, $J = 9.3\text{ Hz}$, 1H), 2.35–2.15 (m, 3H), 1.90–1.75 (m, 3H), 1.68 (sextet, $J = 7.2\text{ Hz}$, 2H), 1.55–1.30 (m, 7H), 0.98 (t, $J = 7.3\text{ Hz}$, 3H), 0.71 (s, 3H); ^{13}C NMR (CDCl_3 , 75 MHz) δ 174.5, 153.5, 138.4, 132.8, 126.7, 115.4, 112.8, 66.0, 55.6, 55.1, 44.3, 43.9, 39.0, 38.6, 29.8, 27.8, 26.7, 24.3, 23.7, 22.3, 13.7, 10.9. Anal. Calcd for $\text{C}_{22}\text{H}_{30}\text{O}_3 \cdot 1/2\text{H}_2\text{O}$: C, 75.18; H 8.89. Found: C, 75.36; H, 8.28.

4.2.3. (20S) 3-(Phenylmethoxy)-19,24-dinorchola-1,3,5(10),16-tetraene (6)

To a solution of **5** (0.20 g, 0.50 mmol) in benzene (10 mL) in a Schlenk flask was added Rh(PPh₃)₃Cl (40 mg, 0.043 mmol). The reaction mixture was cooled with a dry ice–acetone bath, evacuated under high vacuum, and the system refilled to 1 atm with H₂ gas. The mixture was stirred for 7 h at room temperature, and then the solvent was evaporated. The residue was extracted several times with ether, filtered, and concentrated. The residue was purified by column chromatography (SiO₂, hexanes–CH₂Cl₂ = 10:1) to afford **6** (138 mg, 69%) as a colorless solid. Mp 82–83.5 °C, [*a*]_{20D} +67 (*c* 0.74, acetone); ¹H NMR (CDCl₃, 300 MHz) δ 7.46–7.30 (m, 5H), 7.20 (d, *J* = 8.4 Hz, 1H), 6.78 (br d, *J* = 8.4 Hz, 1H), 6.74 (br s, 1H), 5.35 (br s, 1H), 5.04 (s, 2H), 2.94–2.84 (m, 2H), 2.40–2.08 (m, 4H), 2.00–1.87 (m, 3H), 1.65–1.28 (m, 7H), 1.09 (d, *J* = 6.6 Hz, 3H), 0.89 (t, *J* = 7.3 Hz, 3H), 0.83 (s, 3H); ¹³C NMR (CDCl₃, 75 MHz) δ 160.2, 155.9, 137.6, 136.7, 132.9, 128.0, 127.3, 127.0, 125.6, 120.4, 114.4, 111.8, 70.0, 56.4, 47.8, 44.7, 37.8, 35.4, 33.6, 31.3, 30.3, 30.2, 28.2, 27.0, 21.3, 17.1, 12.4. Anal. Calcd for C₂₉H₃₆O: C, 86.95; H, 9.06. Found: C, 86.99; H, 9.12.

4.2.4. (20S) 3-Hydroxy-19,24-dinorchola-1,3,5(10),16-tetraene (7)

Cleavage of the benzyl ether **6** (73 mg, 0.18 mmol) with sodium metal in *n*-butanol was carried out in a fashion similar to the cleavage of **8**. Purification of the residue by column chromatography (SiO₂, hexanes–ethyl acetate gradient = 5:1) gave unreacted starting material (17 mg) followed by **7** (46 mg, 81%) as a colorless solid. Mp 92–95 °C, [*a*]_{20D} +86.3 (*c* 0.32, acetone); ¹H NMR (acetone-*d*₆) δ 7.05 (d, *J* = 8.4 Hz, 1H), 6.56 (dd, *J* = 2.1, 8.4 Hz, 1H), 6.51 (d, *J* = 2.1 Hz, 1H), 5.35 (br s, 1H), 2.82–2.73 (m, 2H), 2.37–2.28 (m, 1H), 2.22–2.05 (m, 2H), 1.97–1.85 (m, 4H), 1.60–1.26 (m, 8H), 1.07 (d, *J* = 7.2 Hz, 3H), 0.87 (t, *J* = 7.5 Hz, 3H), 0.82 (s, 3H); ¹³C NMR (acetone-*d*₆) δ 162.5, 156.7, 139.3, 133.2, 127.7, 122.7, 117.1, 114.7, 58.8, 50.0, 47.1, 40.4, 37.7, 35.8, 33.4, 32.5, 32.2, 30.6, 29.3, 23.2, 19.0, 14.1. Anal. Calcd for C₂₂H₃₀O·1/6H₂O: C, 84.28; H, 9.75. Found: C, 84.28; H, 9.82.

4.2.5. (20S) 3-Hydroxy-19,24-Dinorchola-1,3,5(10),16-tetraen-23-ol (11)

To a solution of **8** (394 mg, 0.947 mmol) in *n*-butanol (20 mL), at 70 °C, was added sodium metal (0.87 g, 38 mmol) in small pieces. After all of the sodium had reacted, the reaction mixture was cooled to room temperature and quenched with water, followed by saturated aqueous NH₄Cl. The reaction mixture was extracted several times with ether, the combined extracts were dried (MgSO₄) and concentrated. The residue was purified by column chromatography (SiO₂, hexanes–ethyl acetate gradient = 4:1 to 2:1) to afford unreacted starting material (91 mg) followed by **11** (150 mg, 49%) as a colorless solid. Mp 174.5–176 °C, [α]_{20D} +77.5 (c 1.50, acetone); ¹H NMR (acetone-*d*₆) δ 8.15 (s, phenol OH), 7.04 (d, *J* = 8.4 Hz, 1H), 6.56 (dd, *J* = 2.7, 8.4 Hz, 1H), 6.51 (d, *J* = 2.7 Hz, 1H), 5.38 (br s, 1H), 3.64–3.52 (m, 3H), 2.84–2.74 (m, 2H), 2.42–2.28 (m, 2H), 2.20–2.08 (m, 1H), 1.96–1.70 (m, 4H), 1.60–1.30 (m, 7H), 1.10 (d, *J* = 7.2 Hz, 3H), 0.82 (s, 3H); ¹³C NMR (acetone-*d*₆) δ 162.8, 156.6, 139.2, 133.0, 127.6, 122.6, 117.0, 114.6, 61.4, 58.7, 49.9, 47.0, 43.0, 40.3, 37.5, 33.2, 32.0, 30.9, 30.5, 29.2, 23.7, 19.0. Anal. Calcd for C₂₂H₃₀O₂: C, 80.94; H, 9.26. Found: C, 80.67; H, 9.32.

4.2.6. 17,23-Epoxy-3-(phenylmethoxy)-19,24-dinorchola-1,3,5(10)-triene (9)

To a solution of **8** (56 mg, 0.14 mmol) in CHCl₃ (2 mL) was added a drop of concentrated HCl. The mixture was allowed to stand stirred for 24 h at room temperature, and then passed through a short column of silica gel using hexanes–ethyl acetate as eluent. Concentration of the eluent gave **9** (50 mg, 89%) as a colorless oil. [α]_{20D} +36 (c 1.0, CH₂Cl₂); ¹H NMR (CDCl₃, 300 MHz) δ 7.46–7.28 (m, 5H), 7.22 (d, *J* = 8.4 Hz, 1H), 6.87 (dd, *J* = 2.7, 8.4 Hz, 1H), 6.73 (d, *J* = 2.7 Hz, 1H), 5.04 (s, 2H), 3.87 (dt, *J* = 4.5, 7.8 Hz, 1), 3.62 (dt, *J* = 6.4, 7.8 Hz, 1H), 2.92–2.82 (m, 2H), 2.38– 1.20 (m, 16H), 1.10 (d, *J* = 6.9 Hz, 3H), 0.74 (s, 3H); ¹³C NMR (CDCl₃, 75 MHz) δ 155.8, 137.6, 136.7, 132.8, 128.2, 127.3, 126.9, 125.8, 114.4, 111.8, 95.5, 70.0, 66.0, 50.0, 48.2, 44.0, 39.3, 36.9, 35.1, 31.3, 31.0, 30.3, 28.1, 26.6, 23.6, 19.0, 15.8. Anal. Calcd for C₂₉H₃₆O₂: C, 83.61; H, 8.71. Found: C, 83.35; H, 8.75.

4.2.7. 17,23-Epoxy-3-hydroxy-19,24-dinorchola-1,3,5(10)-triene (10)

To a solution of **9** (48.9 mg, 0.118 mmol) in methanol/CHCl₃ (1:100, 6 mL) was added 10% Pd on carbon (5.6 mg). The mixture was stirred under H₂ (ca. 46 psi) in a Paar hydrogenation apparatus for 3 h. The catalyst was removed by filtration through filter-aid and the filter bed was washed with copious CH₂Cl₂ and the combined filtrates were concentrated. The residue was purified by chromatography (SiO₂, hexanes–ethyl acetate = 3:1) to afford **10** as a colorless solid (37.8 mg, 99%). Mp 172–174 °C; ¹H NMR (CDCl₃, 300 MHz) δ 7.15 (d, *J* = 8.4 Hz, 1H), 6.62 (dd, *J* = 2.7, 8.4 Hz, 1H), 6.55 (d, *J* = 2.7 Hz, 1H), 3.87 (dt, *J* = 4.5, 7.8 Hz, 1H), 3.60 (dt, *J* = 6.3, 8.1 Hz, 1H), 2.85–2.75 (m, 2H), 2.35–1.20 (m, 16H), 1.07 (d, *J* = 6.9 Hz, 3H), 0.70 (s, 3H); ¹³C NMR (CDCl₃, 75 MHz) δ 153.3, 138.6, 133.2, 126.6, 115.4, 112.7, 96.0, 66.1, 50.0, 48.2, 43.9, 39.3, 36.8, 35.0, 31.2, 30.8, 30.0, 27.9, 26.4, 23.4, 18.8, 15.6. Anal. Calcd for C₂₂H₃₀O₂·1/4H₂O: C, 79.83; H 9.29. Found: C, 80.12; H, 9.33.

4.2.8. (20S) 3-Hydroxy-19,24-dinorchola-1,3,5(10),16-tetraen-23-al (12)

To a solution of **11** (100 mg, 0.296 mmol) in THF (4 mL) was added a solution of ethyl magnesium bromide in THF (0.67 mL, 1.0 M, 0.67 mmol). The solution was stirred at room temperature for 15 min, and then solid 1,1'-(azodicarbonyl)dipiperidine (0.17 g, 0.67 mmol) was added. The reaction mixture was stirred for 1 h, and then quenched with saturated aqueous NH₄Cl and extracted several times with ether. The combined ethereal extracts were dried (MgSO₄), concentrated and the residue was purified by column chromatography (SiO₂, hexanes–ethyl acetate = 5:1) to afford **12** as a colorless solid (66 mg, 66%). Mp 168.5–171 °C, [*a*]_D²⁰ +78 (c 0.80, acetone); ¹H NMR (acetone-*d*₆, 300 MHz) δ 9.66 (t, *J* = 2.1 Hz, 1H), 7.06 (d, *J* = 8.4 Hz, 1H), 6.57 (dd, *J* = 2.5, 8.4 Hz, 1H), 6.51 (d, *J* = 2.5 Hz, 1H), 5.46 (br s, 1H), 2.90–2.75 (m, 4H), 2.62 (ddd, *J* = 1.8, 5.7, 16.2 Hz, 1H), 2.44–2.30 (m, 2H), 2.26–2.10 (m, 2H), 1.98–1.86 (m, 3H), 1.60–1.34 (m, 5H), 1.16 (d, *J* = 7.2 Hz, 3H), 0.88 (s, 3H); ¹³C NMR (acetone-*d*₆, 75 MHz) δ 203.2, 161.4, 156.8, 139.5, 133.3, 127.9, 124.6, 117.2, 114.8, 59.2, 53.1, 50.2, 47.2, 40.5, 37.7, 33.6, 32.3,

30.7, 29.7, 29.4, 23.8, 19.3. Anal. Calcd for C₂₂H₂₈O₂: C, 81.44; H, 8.70. Found: C, 81.21; H, 8.54.

4.2.9. 17,23-Epoxy-3-hydroxy-19-norchola-1,3,5(10)-triene (13)

To a solution of **12** (45.9 mg, 0.142 mmol) in THF (7 mL) at 0 °C was added a solution of methyl magnesium bromide in ether (0.10 mL, 3.0 M, 0.30 mmol). The reaction mixture was stirred for 3 h, and then quenched with saturated aqueous NH₄Cl (15 mL). The mixture was extracted several times with CH₂Cl₂ and the combined extracts were dried (MgSO₄) and concentrated. The residue was purified by chromatography (SiO₂, hexanes–ethyl acetate = 5:1) to afford **13** as a colorless solid (44 mg, 92%). Analysis of the product by ¹H NMR spectroscopy indicated this to be a 1:1 mixture of diastereomers. Mp 248–251 °C, ¹H NMR (CDCl₃, 300 MHz) δ 7.15 (d, *J* = 8.4 Hz, 1H), 6.62 (dd, *J* = 2.7, 8.4 Hz, 1H), 6.56 (d, *J* = 2.7 Hz, 1H), 4.18–4.07 (m, 1H), 3.85–3.74 (m, 1H), 2.85–2.75 (m, 2H), 2.35–1.20 (m, 15H), 1.23 & 1.20 (2 × d, *J* = 5.7 Hz, 3H total), 1.07 & 1.05 (2 × d, *J* = 6.9 Hz, 3H), 0.72 & 0.66 (2 × s, 3H total); ¹³C NMR (CDCl₃, 75 MHz) δ 153.3, 138.6, 133.2, 126.6, 115.4, 112.7, 97.1 [95.8], 73.6 [71.3], 49.85 [49.80], 48.8, 47.1, 45.4, 43.9 [43.8], 43.5, 39.3 [39.2], 36.2, 34.5, 32.3, 31.2 [30.9], 30.6 [30.1], 27.8, 26.5 [26.4], 23.5 [23.4], 21.6, 19.2 [18.9], 16.3 [14.9]. Anal. Calcd for C₂₃H₃₂O₂·1/2H₂O: C, 79.04; H, 9.52. Found: C, 79.34; H, 9.57.

4.2.10. *cis*- and *trans*-4-(4'-Hydroxycyclohexyl)phenol (14)

To a solution of 4-(4'-hydroxyphenyl)cyclohexanone (50 mg, 0.26 mmol) in methanol (1 mL) was added NaBH₄ (15 mg, 4.0 mmol). The reaction mixture was stirred for 30 min, and then diluted with water. The mixture was extracted several times with ethyl acetate and the combined extracts were concentrated and purified by column chromatography (SiO₂, hexanes–ethyl acetate = 2:1) to afford *cis*-**14** (5.0 mg, 10%) followed by *trans*-**15** (43 mg, 86%) both as colorless solids. *Cis*-**14**: ¹H NMR (CD₃OD, 400 MHz) δ 7.04–6.69 (AA'BB', *J*_{AB} = 8.8 Hz, 4H), 4.02 (narrow t, *J* = 2.8 Hz, 1H), 2.50–2.40 (m, 1H), 1.91–1.79 (m, 4H), 1.69–1.52 (m, 4H); ¹³C NMR (CD₃OD, 75 MHz) δ 156.5, 140.1, 128.8, 116.1, 66.5, 44.5, 34.0, 29.4. *Trans*-**15**: ¹H NMR (CD₃OD, 400 MHz) δ 7.01–6.68 (AA'BB', *J*_{AB} = 8.4 Hz, 4H), 3.58 (tt, *J*

= 4.4, 10.6 Hz, 1H), 2.39 (tt, $J = 3.5, 11.8$ Hz, 1H), 2.06–1.99 (m, 2H), 1.87–1.79 (m, 2H), 1.56–1.33 (m, 4H).

4.2.11. 4-(4-Hydroxyphenyl)-cyclohexanone oxime (16)

To a solution of 4-(4'-hydroxyphenyl)cyclohexanone (50 mg, 0.26 mmol), hydroxylamine hydrochloride (36.6 mg, 0.526 mmol) in ethanol (5 mL) was added Amberlyst (56 mg). After stirring for 2 h, the mixture was filtered, and the filtrate concentrated. The residue was partitioned between water and ethyl acetate, and the organic layer was concentrated and dried to give (±)-**16** (44 mg, 82%) as a colorless solid. Mp 172–175 °C. ^1H NMR (CD_3OD , 400 MHz) δ 7.03–6.69 (AA'BB', $J_{AB} = 8.8$ Hz, 4H), 4.02 (narrow t, $J = 2.8$ Hz, 1H), 2.0–2.40 (m, 1H), 1.91–1.79 (m, 4H), 1.69–1.52 (m, 4H); ^{13}C NMR (CD_3OD , 75 MHz) δ 161.0, 156.8, 138.4, 128.7, 116.3, 44.3, 36.0, 34.7, 33.0, 25.2. HRMS (ESI): m/z calcd for $\text{C}_{12}\text{H}_{15}\text{NO}_2 + \text{Na}^+$ $[\text{M} + \text{Na}]^+$ 228.0995, found 228.0997.

4.2.12. cis-1-Hydroxymethyl-4-(4'-hydroxyphenyl)-cycloheptane (18)

To a solution of (±)-**17** (75 mg, 0.35 mmol) in methanol (15 mL) in a heavy walled reaction vessel, was added a catalytic amount of 20% Pd/C. The mixture was stirred under H_2 pressure (45 psi) for 75 min and then the reaction mixture was filtered through the pad of celite. The filtrate was concentrated and the residue was purified by column chromatography (SiO_2 , hexanes–ethyl acetate = 65:35) to afford (±)-**18** (38 mg, 50%) as a colorless solid. Mp 60–61 °C; ^1H NMR (CDCl_3 , 300 MHz) δ 7.06 and 6.75 (AA'BB', $J_{AB} = 9.0$ Hz, 4H), 3.48 (d, $J = 6.3$ Hz, 1H), 2.59–2.58 (m, 1H), 1.95–1.08 (m, 13H); ^{13}C NMR (CD_3OD , 75 MHz) δ 127.9, 115.3, 68.6, 46.1, 41.4, 38.8, 33.1, 31.6, 28.5, 27.5. HRMS (ESI): m/z calcd for $\text{C}_{14}\text{H}_{20}\text{O}_2 + \text{Na}^+$ $[\text{M} + \text{Na}]^+$ 243.1356, found 243.1356.

4.2.13. 5-[(1E)-2-(4-Hydroxyphenyl)ethenyl]-2-furanmethanol (20)

A solution of methyl 5-bromo-2-furanoate (1.03 g, 5.02 mmol), 4-acetoxystyrene (0.97 g, 6.0 mmol), palladium acetate (0.01 g, 0.05 mmol), tri-*o*-tolylphosphine (0.03 g, 0.2 mmol), and triethylamine (3

mL) was heated under nitrogen in a sealed heavy-walled Pyrex tube at 100 °C for 24 h. The reaction mixture was cooled, diluted with water and dichloromethane. The dichloromethane layer was separated, washed with water, and dried (MgSO₄), and the residue was purified by column chromatography (SiO₂, hexanes–ethyl acetate = 4:1) to afford **19** (350 mg, 24%), a pale yellow solid. Mp 110.5–112 °C; ¹H NMR (CDCl₃, 300 MHz) δ 7.51 (d, *J* = 8.1, 2H), 7.27 (d, *J* = 16.5 Hz, 1H), 7.20 (d, *J* = 3.6 Hz, 1H), 7.10 (d, *J* = 8.1 Hz, 2H), 6.86 (d, *J* = 16.5 Hz, 1H), 6.45 (d, *J* = 3.6 Hz, 1H), 3.92 (s, 3H, OMe), 2.32 (s, 3H, OAc). This product was used in the next step without further characterization. To a solution of diester (50 mg, 0.17 mmol) in anhydrous ether (1 mL) at 0 °C, was slowly added a solution of lithium aluminium hydride (0.52 mL, 1.0 M in THF, 0.52 mmol). Solution was stirred for 3 h at 0 °C and then saturated aqueous sodium bicarbonate (2 mL) was added follow by dilute sodium hydroxide. The mixture was warmed to room temperature, extracted several times with ethyl acetate. The combined extracts were dried (MgSO₄), concentrated and the residue was purified by column chromatography (SiO₂, hexanes–ethyl acetate = 1:1) gave **20** (28 mg, 74%) as a colorless solid. Mp 129–131 °C; ¹H NMR (acetone-*d*₆, 300 MHz) δ 8.59 (br s, 1H), 7.40 (d, *J* = 9.0 Hz, 2H), 6.97– 6.79 (m, 4H), 6.30 (s, 2H), 4.57 (br s, 2H), 3.05 (br s, 1H); ¹³C NMR (acetone-*d*₆, 75 MHz) δ 158.2, 155.9, 154.1, 129.7, 128.6, 127.4, 116.5, 114.9, 109.9, 109.4, 57.4. HRMS (ESI): *m/z* calcd for C₁₃H₁₂O₃+Na⁺ [M+Na]⁺ 239.0679, found 239.0681.

4.3. Fluorescence polarization

The assay was developed based on a commercially available kit from Invitrogen.¹⁵ Assays were run on a BMG POLARstar Galaxy reader with acquisition parameters as follows: 200 flashes, positioning delay 1.0 s, *K* factor ≤ 1.1 and ≥ 0.9, excitation filter of 485 ± 5 nm and emission filter of 520 ± 15 nm. For the IC₅₀ determinations the [ER-α] was 30 nM and the [FITC-estradiol tracer] ([Tr]) was 10 nM. Sample volume was 150 μL. For each experiment the polarization was calibrated with a sample of FITC set at 20 mP. All proper blanks were used, including water for the FITC samples and blank samples containing only 30 nM ERα protein for the remaining data points. All protein samples contained 1% DMSO-*d*₆, the maximum amount tolerated as stated by the supplier of the ERα protein, Invitrogen, to

ensure the solubility of all hydrophobic compounds investigated. The K_d of the FITC-tagged estradiol for ER- α was determined by non-linear least squares fitting of the titration curve data to the following equation (where Tr_0 is the F-E2 tracer):

$$\Delta FP = \frac{FP_{max}}{2[Tr_0]} (K_d + [Tr_0] + [ER\alpha]) - \{(K_d + [Tr_0] + [ER\alpha])^2 - 4[Tr_0]\} + [ER\alpha]$$

4.4. Cell-based ER α and ER β assays

ER α and ER β assay kits for cell-based assays (Indigo Biosciences) allowed for investigation into the functional activity (i.e., agonist and/or antagonist) of the ligands identified to bind based on the initial fluorescence polarization displacement assay. Briefly, the cells contained a luciferase reporter gene that was functionally linked to either the ER α or ER β -responsive promoter. By quantifying the luciferase expression via luminescence, the change in ER activity could be quantified. 1–2 mM stocks of the ligands were prepared in DMSO- d_6 and diluted to final concentrations ranging from 3.2 nM to 2 μ M, using the Compound Screening Medium provided in the kit. For the agonist assay, the cells were prepared by warming to 37 °C, plated, then the chemicals added. For the antagonist assay, the cells were prepared as above with the addition of E2 (for ER α 3.2 nM was added, approximating an IC₇₅; and, for ER β 160 pM was added, approximating an IC₈₀). The cells were then plated, and the chemicals added. All plates were incubated in a cell culture incubator at 37 °C and 5% CO₂ for 22 h. Each assay was performed in duplicate. Luminescence was characterized after removal of the incubating media and introduction of the Detection Substrate using a Molecular Devices SpectraMax M5 microplate reader. Data was fitted using GraphPad Prism and fit to the dose-response (four parameter) equation as follows.

$$y = \frac{\text{bottom} - (\text{top} - \text{bottom})}{(1 + 10^{(\log IC_{50} - x) \text{Hillslope}})}$$

4.5. Molecular docking

Ligand structures were drawn in PC Spartan Plus (Wavefunction) and three dimensional (3D) conformation was then optimized using semiempirical Austin Model 1 (AM1) calculations. Since compound **13** was afforded as a pair of diastereomers both were modeled and docked. The AM1 calculations provided geometries and bond distances for subsequent docking. AutoDock Tools (ADT) was used prepare the ligand files according to AutoDock requirements and assign Gasteiger charges.

The ER α receptor for agonist (pdb code 1ere)⁴ and antagonist (pdb code 1err)³² conformations were prepared for docking calculations using the 'A' chain. The ER β receptor for agonist (pdb code 2jj3)³³ and antagonist (pdb code 1l2j)³⁴ conformations were prepared for docking calculations using the 'A' chain. ADT was used to further prepare the ER receptor files by adding hydrogen atoms and adding partial charges to each atom of the protein. The grid box was centered on the co-crystallized ligand, drawn to a box to incorporate amino acids Arg394, Glu353, and His524 for ER α and Arg346, Glu305, and His475 for ER β , then the estradiol ligand was removed.³⁵ AutoDock (v. 4.2) calculations were performed with default parameters, except with 100 genetic algorithmic runs and 2,500,000 evaluations per run.³⁵⁻³⁹

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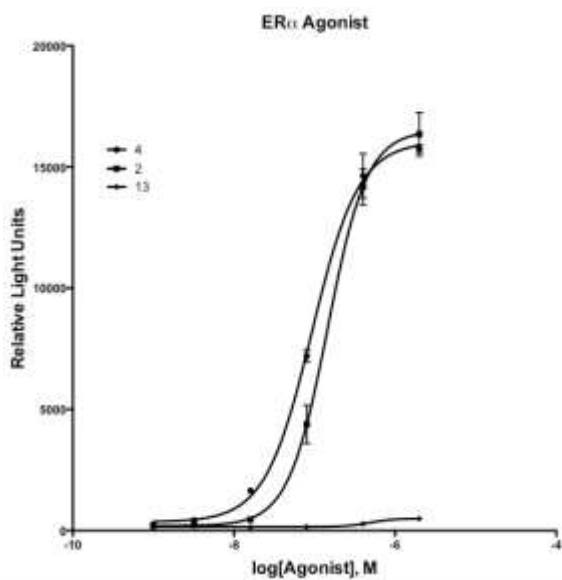
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Footnotes

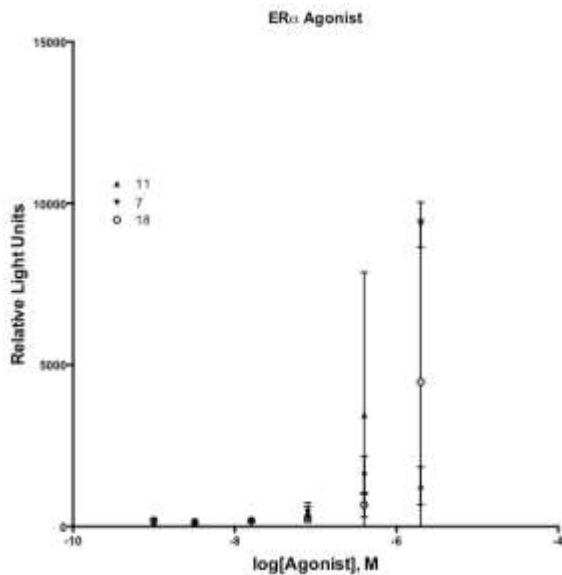
A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.bmc.2013.11.024>.

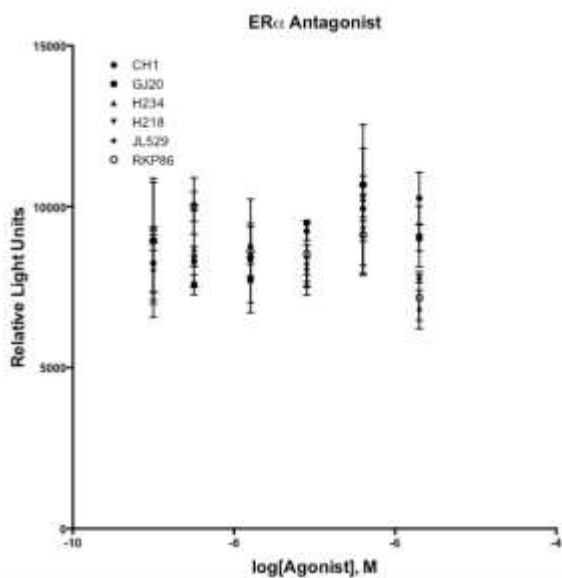
Supplementary Material 1



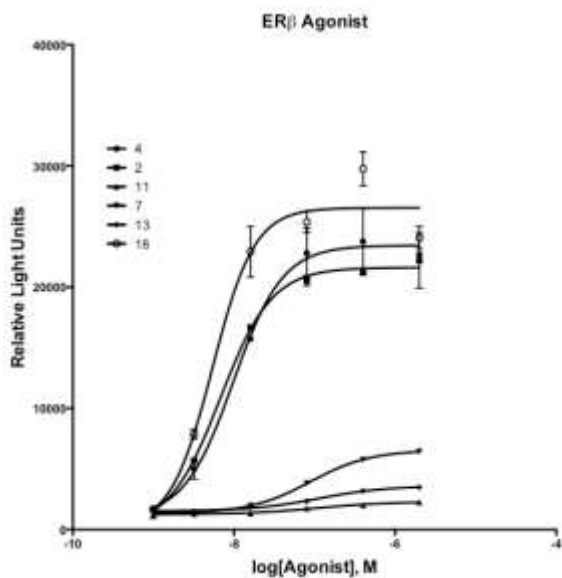
Supplementary Figure S1. Cell-based ER α assay data including regression for ligands that showed agonist activity.



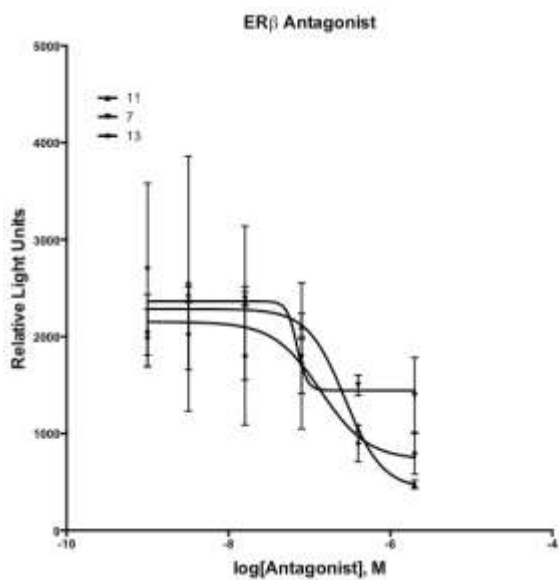
Supplementary Figure S2. Cell-based ER α agonist assay data for chemicals without sufficient quality data to determine activity.



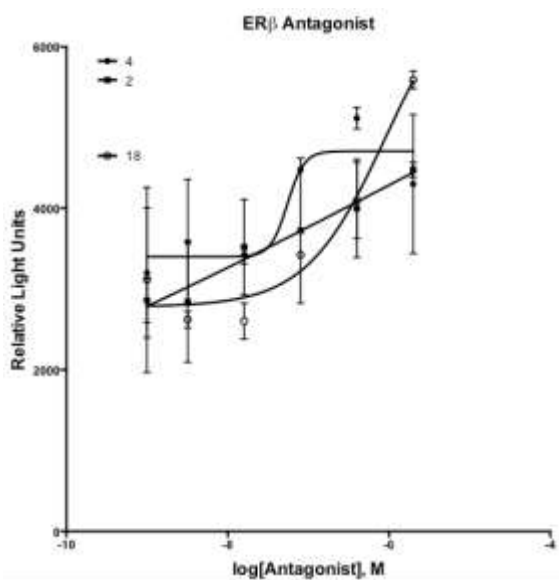
Supplementary Figure S3. Cell-based ER α antagonist assay data for chemicals without sufficient quality data to determine activity.



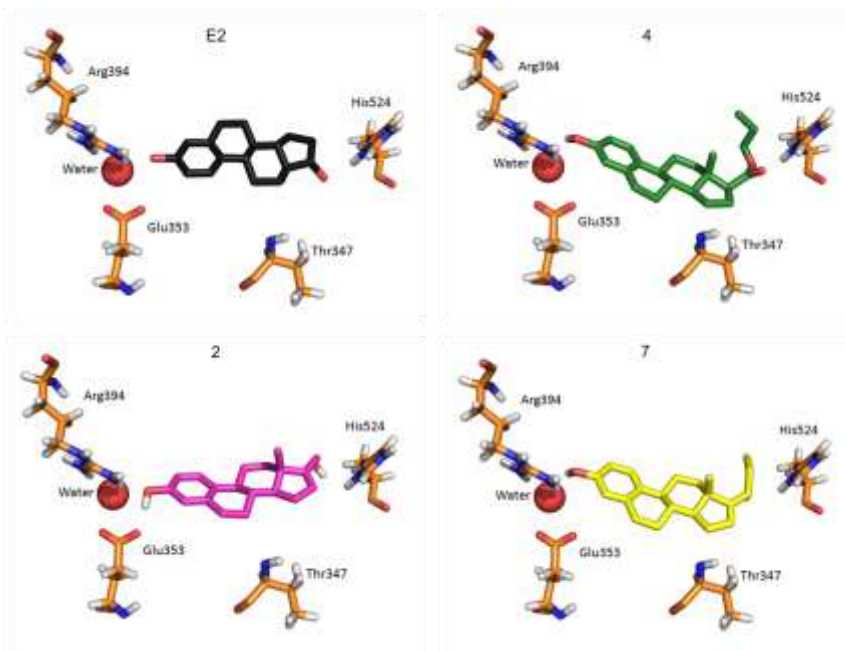
Supplementary Figure S4. Cell-based ER β agonist assay data for ligands that showed antagonist activity



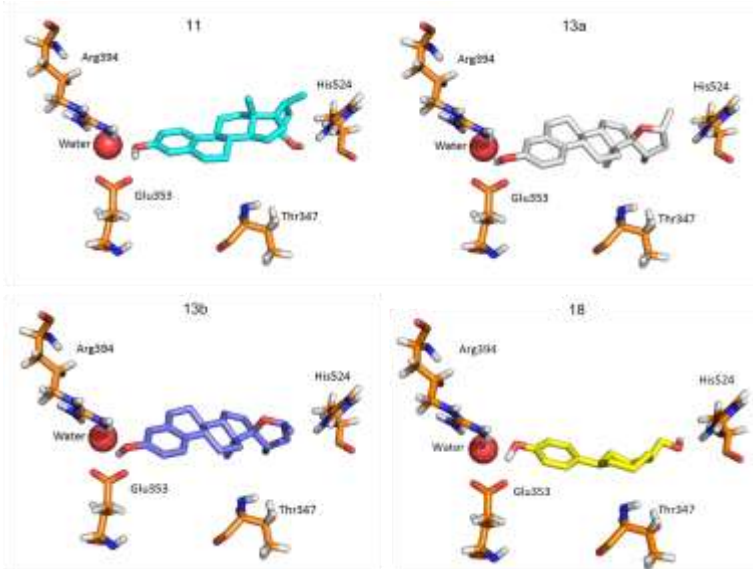
Supplementary Figure S5. Cell-based ER β assay data for chemicals that showed antagonist activity.



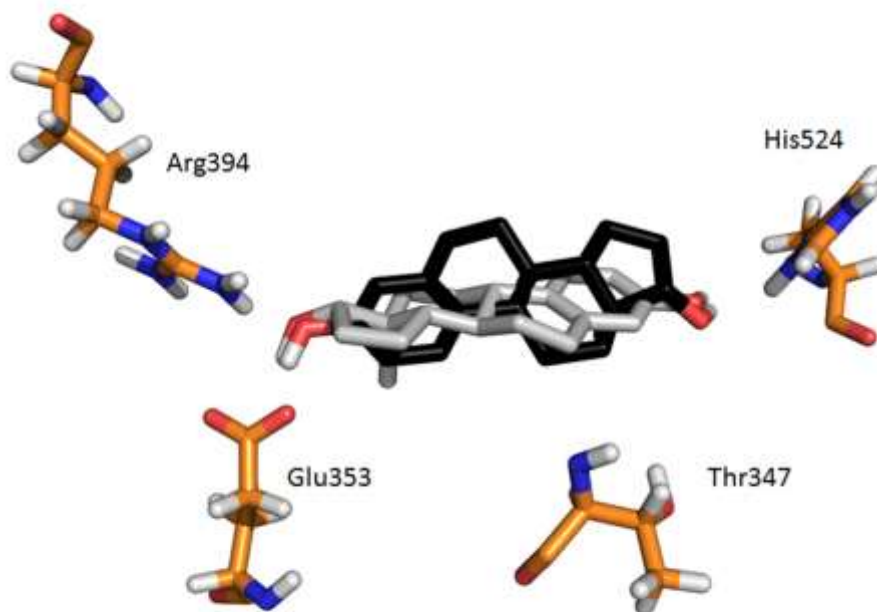
Supplementary Figure S6. Cell-based ER β assay data for chemicals that did not display antagonist activity.



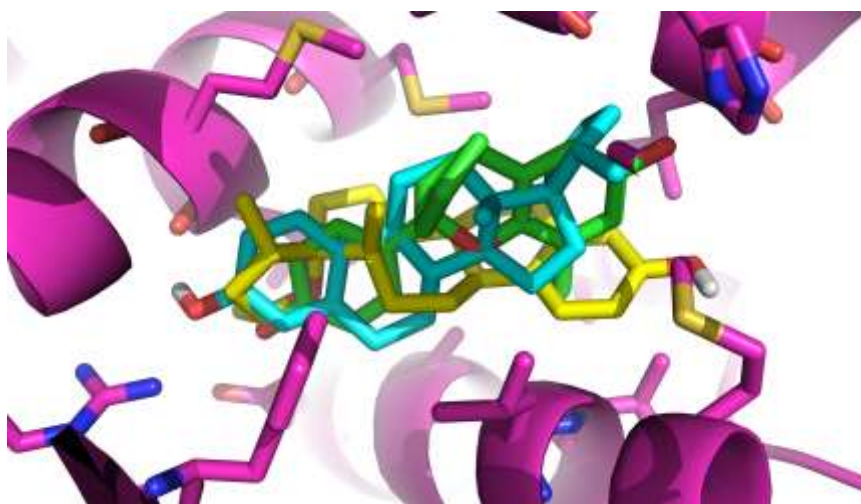
Supplementary Figure S7. Lowest energy docking poses for the ERα-binding compounds identified using fluorescence polarization. PDB file 1ere, chain A was used as the receptor to investigate the predicted affinity for binding in the ERα agonist conformation. Note: E2 is estradiol and provided for comparison.



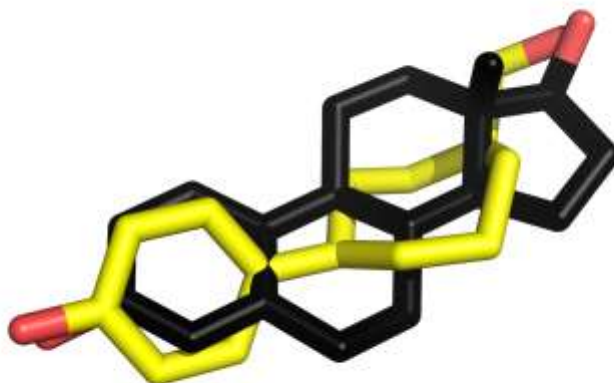
Supplementary Figure S8. Lowest energy docking poses for the ERα-binding compounds identified using fluorescence polarization. PDB file 1ere, chain A was used as the receptor to investigate the predicted affinity for binding in the ERα agonist conformation. Note: chemical 13 was docked using both enantiomers from the racemic mixture.



Supplementary Figure S9. Estradiol docked into PDB file 1ere, chain A with removal of all water molecules. Black is the binding mode associated with the crystal structure (normal). Gray is a 180° rotation of the estradiol (reversed).



Supplementary Figure S10. ERβ agonist conformation (purple) with cocrystalized ligand (green) and docking pose predictions of **10** (blue) and **13** (yellow).



Supplementary Figure S11. Overlay of estradiol (black) and **18** (yellow).

Supplementary Table S1. Docking results for the agonist conformation of ER α in the absence of all water molecules.

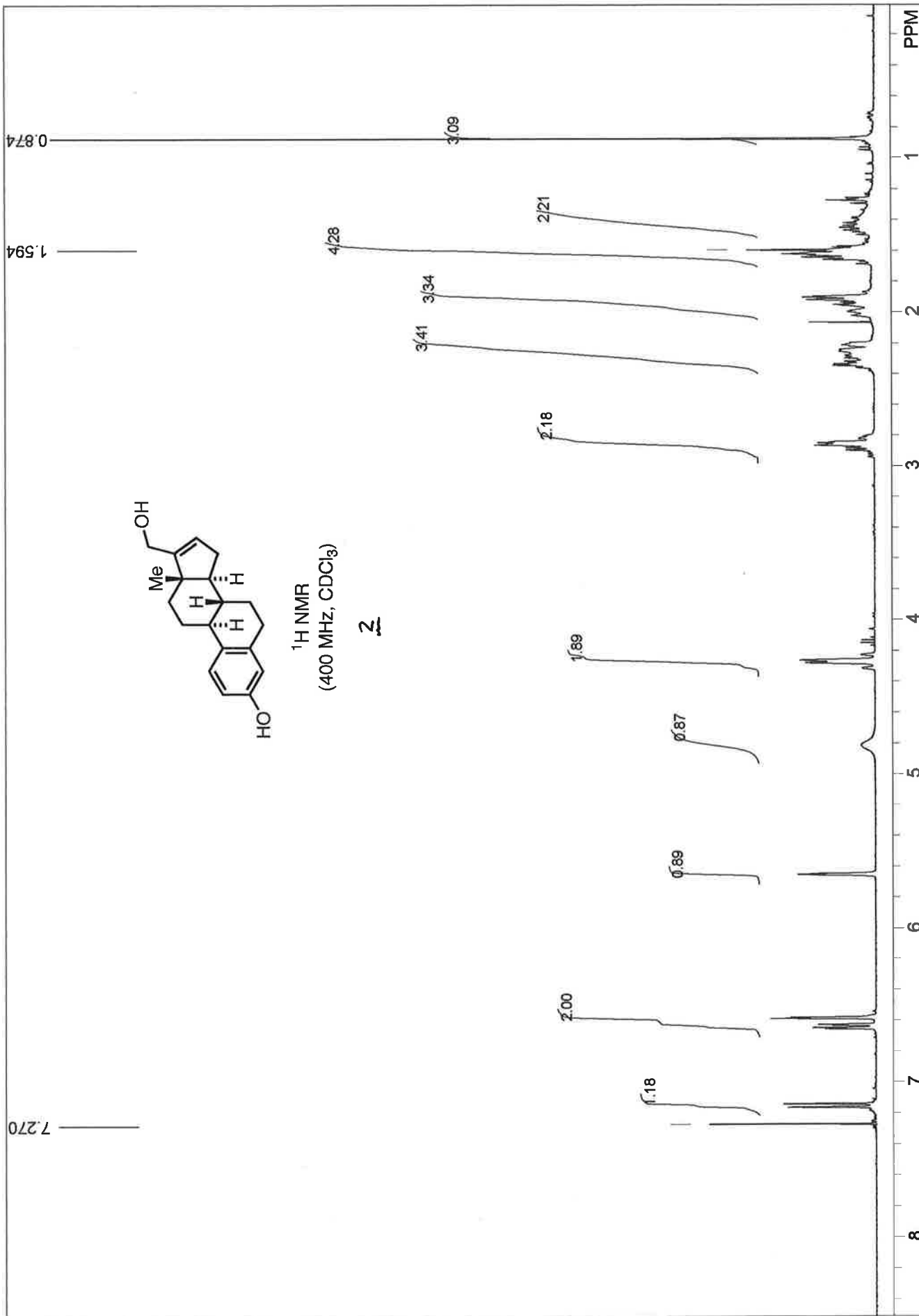
Compound	Number of Clusters (2.0Å rmsd)	Lowest Energy Cluster Population	Calculated Binding Energy (kcal mol ⁻¹)	Mode
estradiol	2	69	-10.74	reversed
estradiol		31	-10.72	normal
4	2	64	-11.09	reversed
4		36	-10.71	normal
2	1	100	-10.98	reversed
7	2	56	-9.93	reversed
7		44	-9.79	normal
11	3	69	-10.35	reversed
11		29	-9.28	normal
11		2	-9.16	reversed
10	2	96	-9.48	reversed
10		4	-9.08	normal
13a	1	100	-7.44	normal
13b	1	100	-9.13	reversed
17	3	22	-7.27	reversed
17		76	-7.21	reversed
17		2	-7.12	normal
20	1	100	-7.57	reversed
18	2	85	-7.42	reversed
18		15	-7.34	normal
14	2	97	-6.71	normal
14		3	-6.39	reversed
15	2	73	-6.85	normal
15		27	-6.77	reversed
16	3	71	-7.42	reversed
16		28	-7.33	normal
16		1	-7.17	normal

Supplementary Table S2. Docking results for the agonist conformation of ER α in the presence of a single water molecule near Arg294 and Glu353 as observed in the crystal structure. Chemicals 20 and 14 were not predicted to bind similarly to the normal or reversed modes as otherwise noted.

Compound	Number of Clusters (2.0Å rmsd)	Lowest Energy Cluster Population	Calculated Binding Energy (kcal mol ⁻¹)	Mode
estradiol	1	100	-10.36	normal
4	2	97	-10.29	normal
2	2	42	-10.16	reversed
2	2	58	-9.82	normal
11	1	100	-9.80	normal
7	1	100	-9.74	normal
10	1	100	-8.82	normal
13b	1	100	-8.73	normal
13a	1	100	-8.39	normal
4	2	3	-7.73	reversed
18	2	72	-7.56	reversed
18	2	28	-7.46	normal
17	2	13	-7.46	reversed
17	2	87	-7.37	normal
16	2	97	-7.27	normal
15	2	73	-7.00	reversed
16	2	3	-6.94	reversed
20	4	76	-6.93	other
15	2	27	-6.85	normal
14	3	79	-6.41	other

NOT THE PUBLISHED VERSION; this is the author's final, peer-reviewed manuscript. The published version may be accessed by following the link in the citation at the bottom of the page.

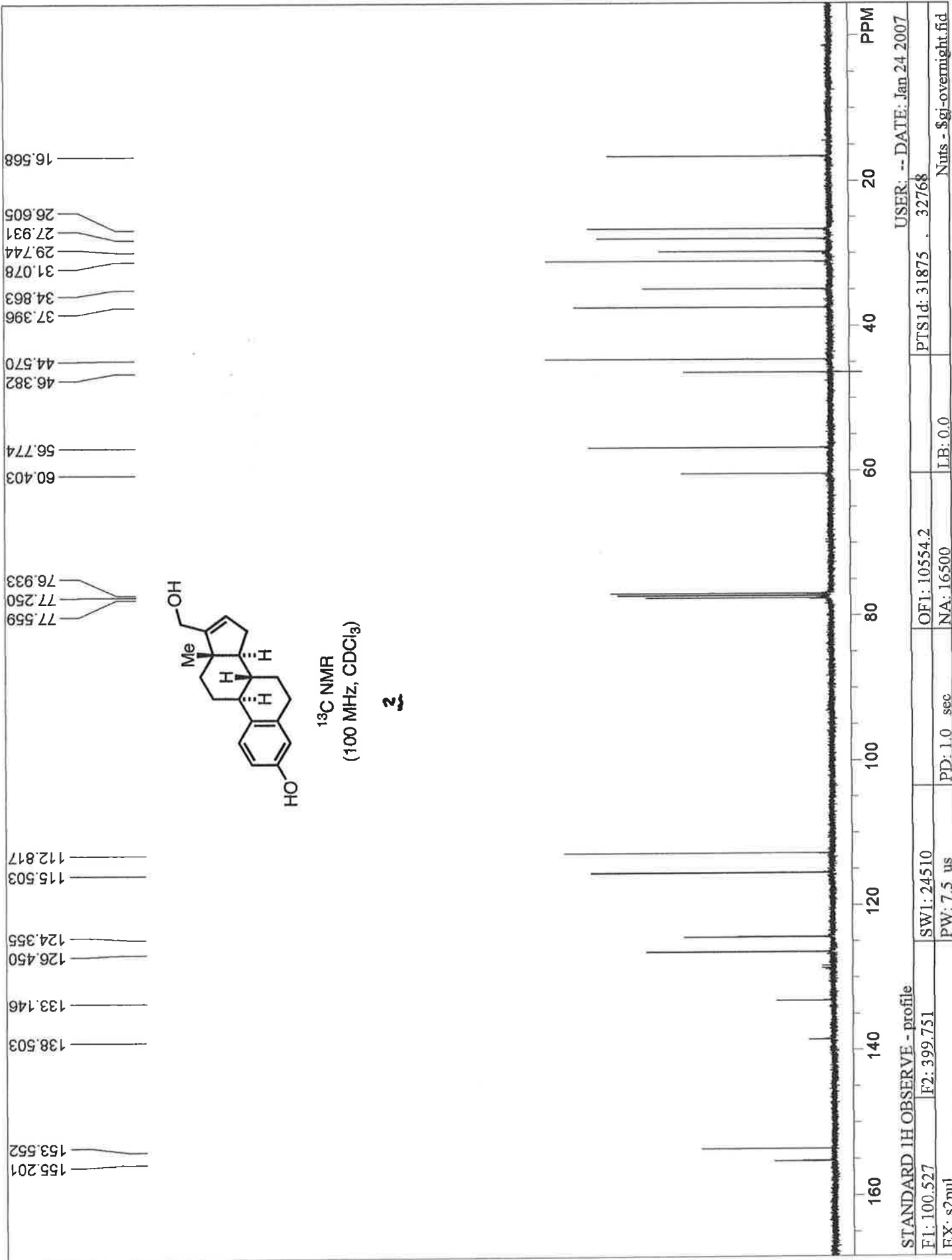
Supplementary Material 2



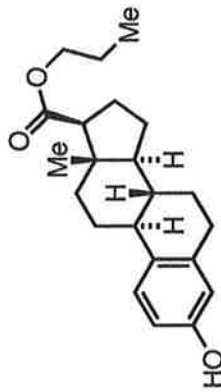
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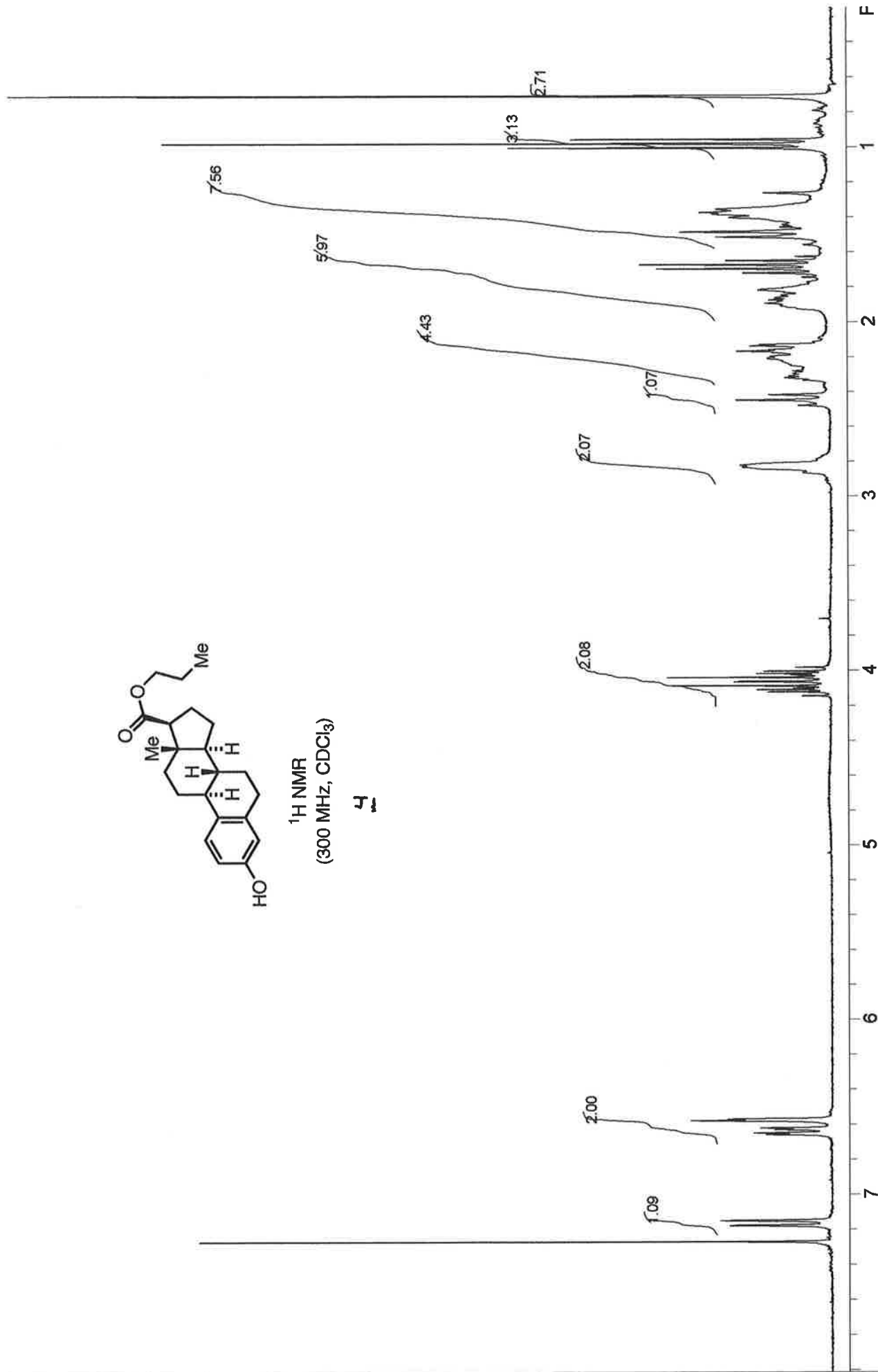


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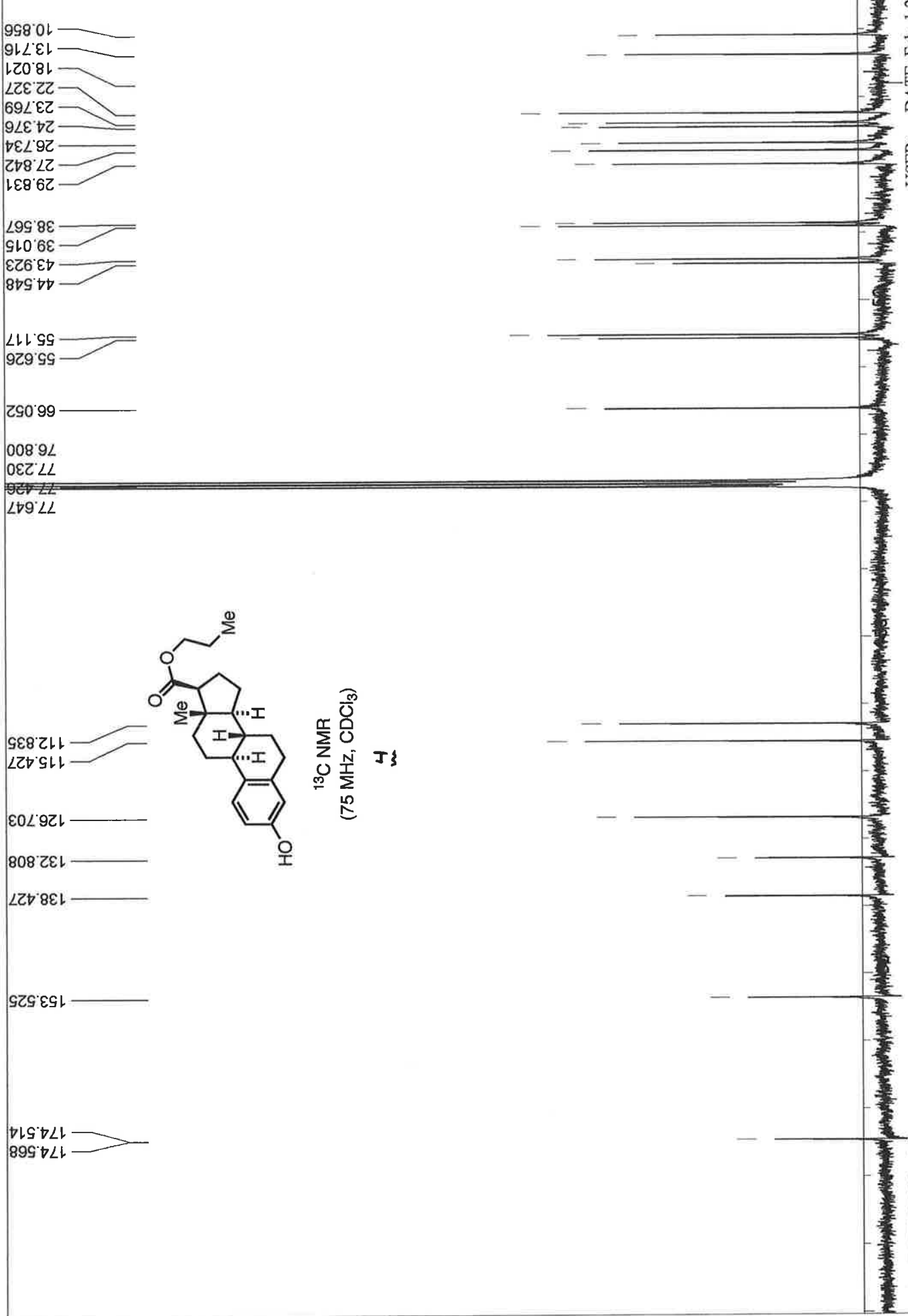


¹H NMR
(300 MHz, CDCl₃)

4



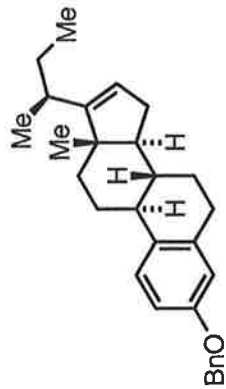
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 EX: s2pul PW: 6.3 us PD: 1.0 sec OF1: 1803.7 NA: 8
 USER: -- DATE: Feb 24 2009 PTS1d: 14409 I6384
 Nuts - SCH-Raney-Nickel-Test LB: 0.0



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13.716
18.021
22.327
23.769
24.376
26.734
27.842
29.831
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39.015
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77.230
77.426
77.647

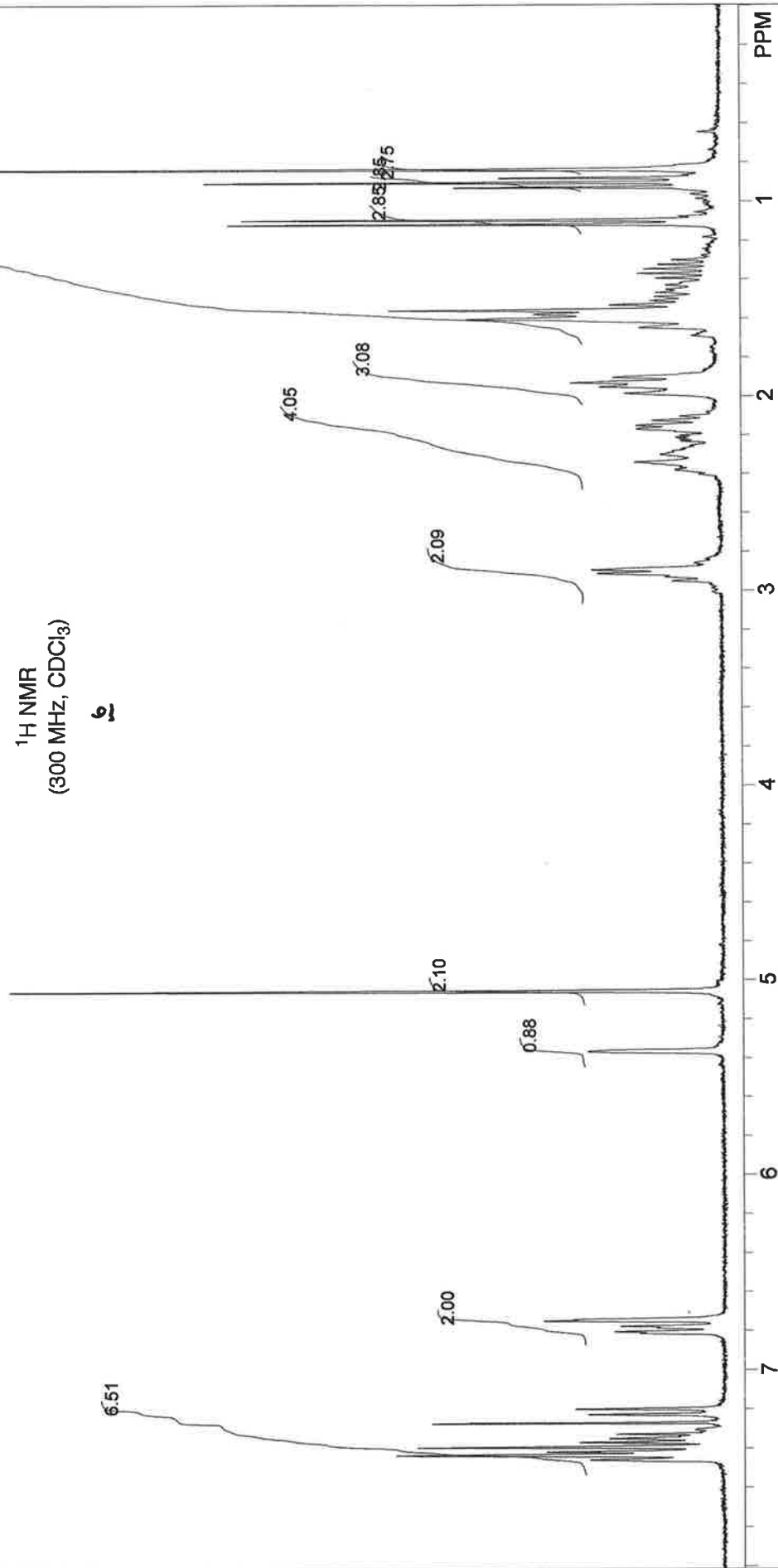
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138.427
153.525
174.514
174.568

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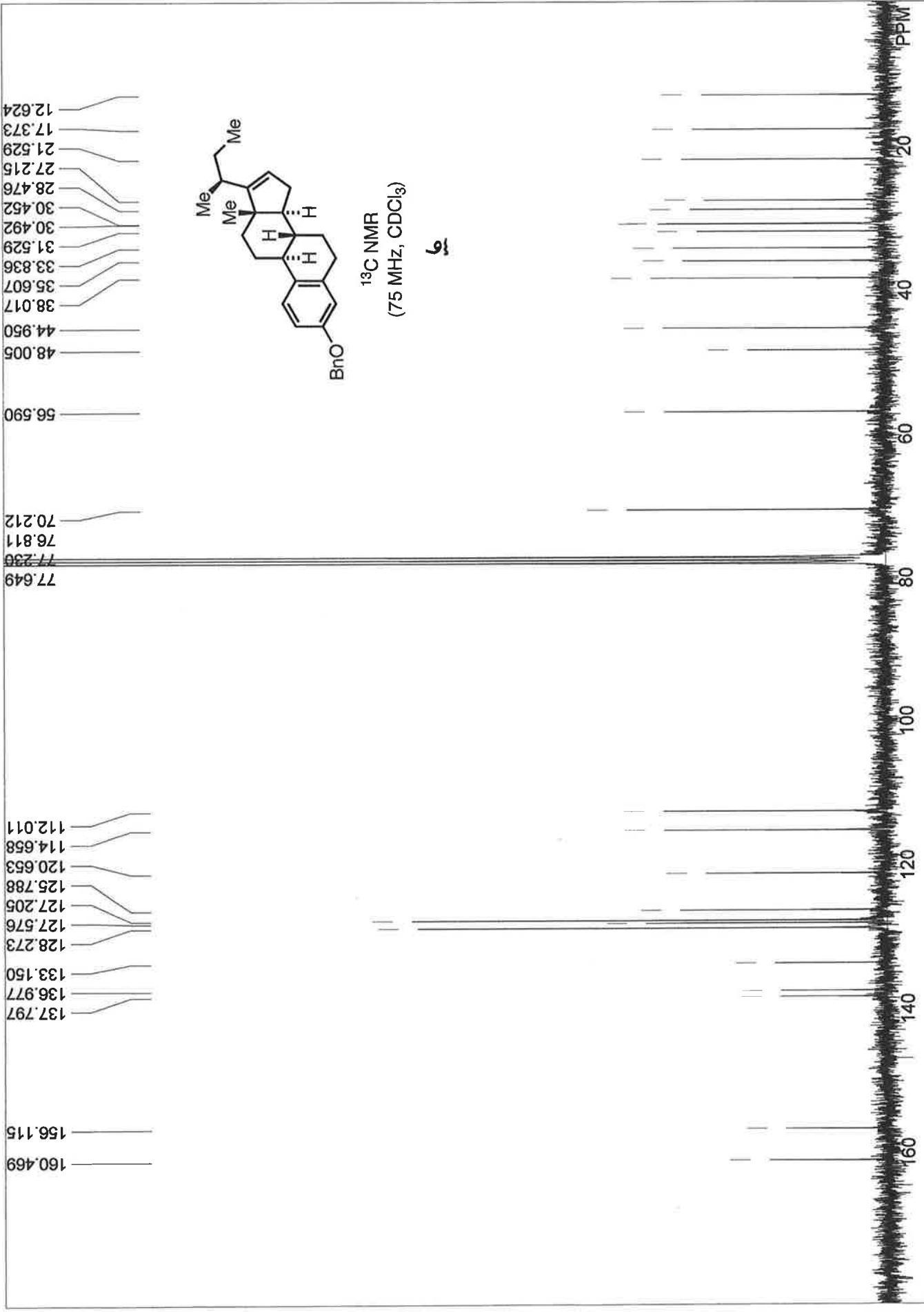


¹H NMR
(300 MHz, CDCl₃)

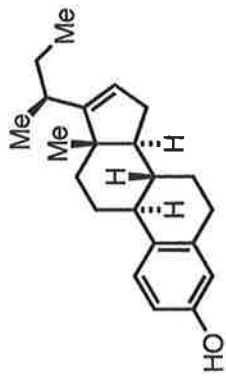
6



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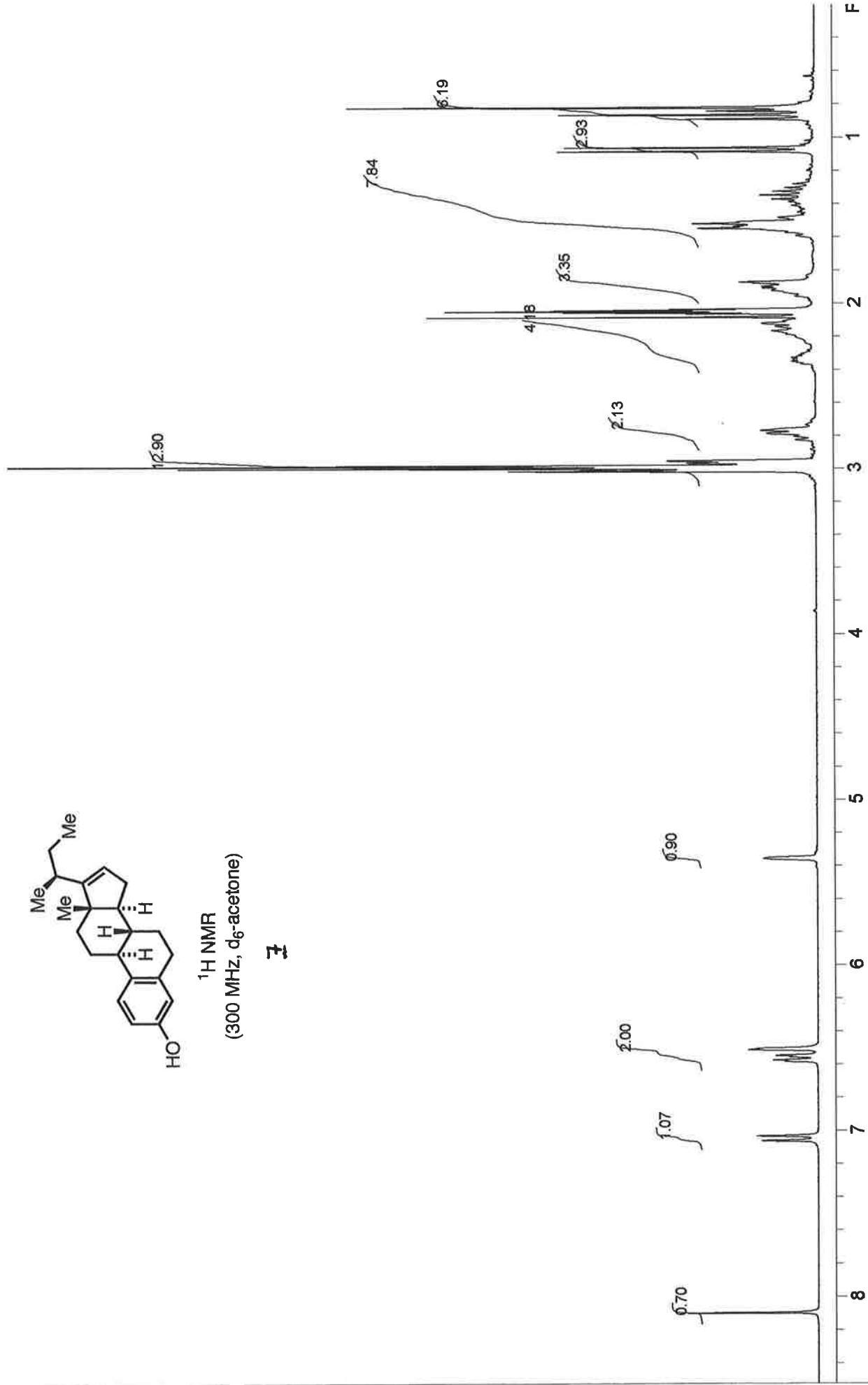


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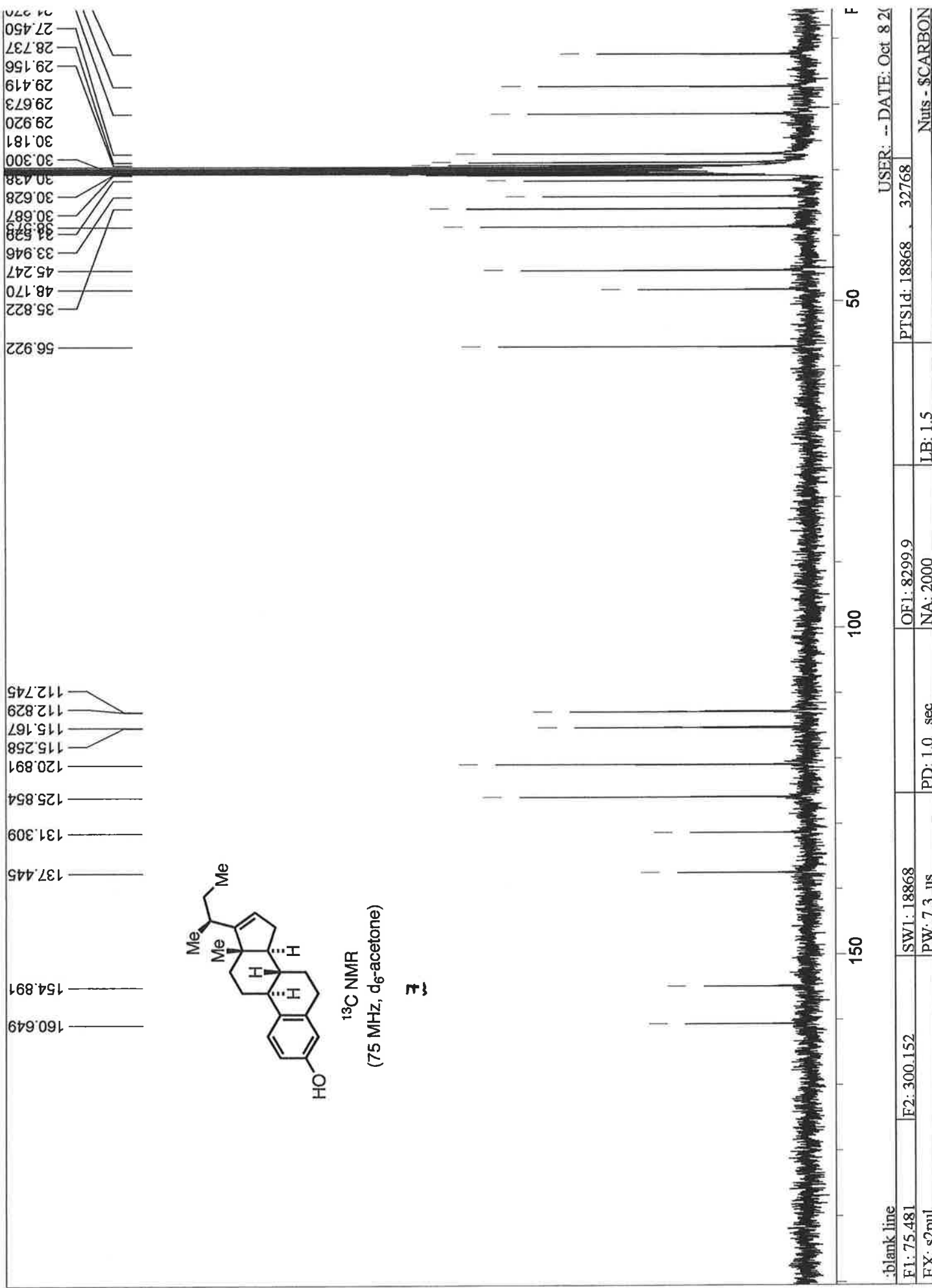


¹H NMR
(300 MHz, d₆-acetone)

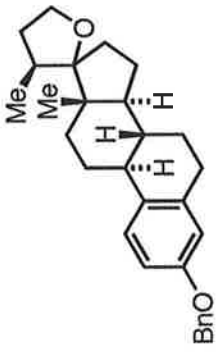
7



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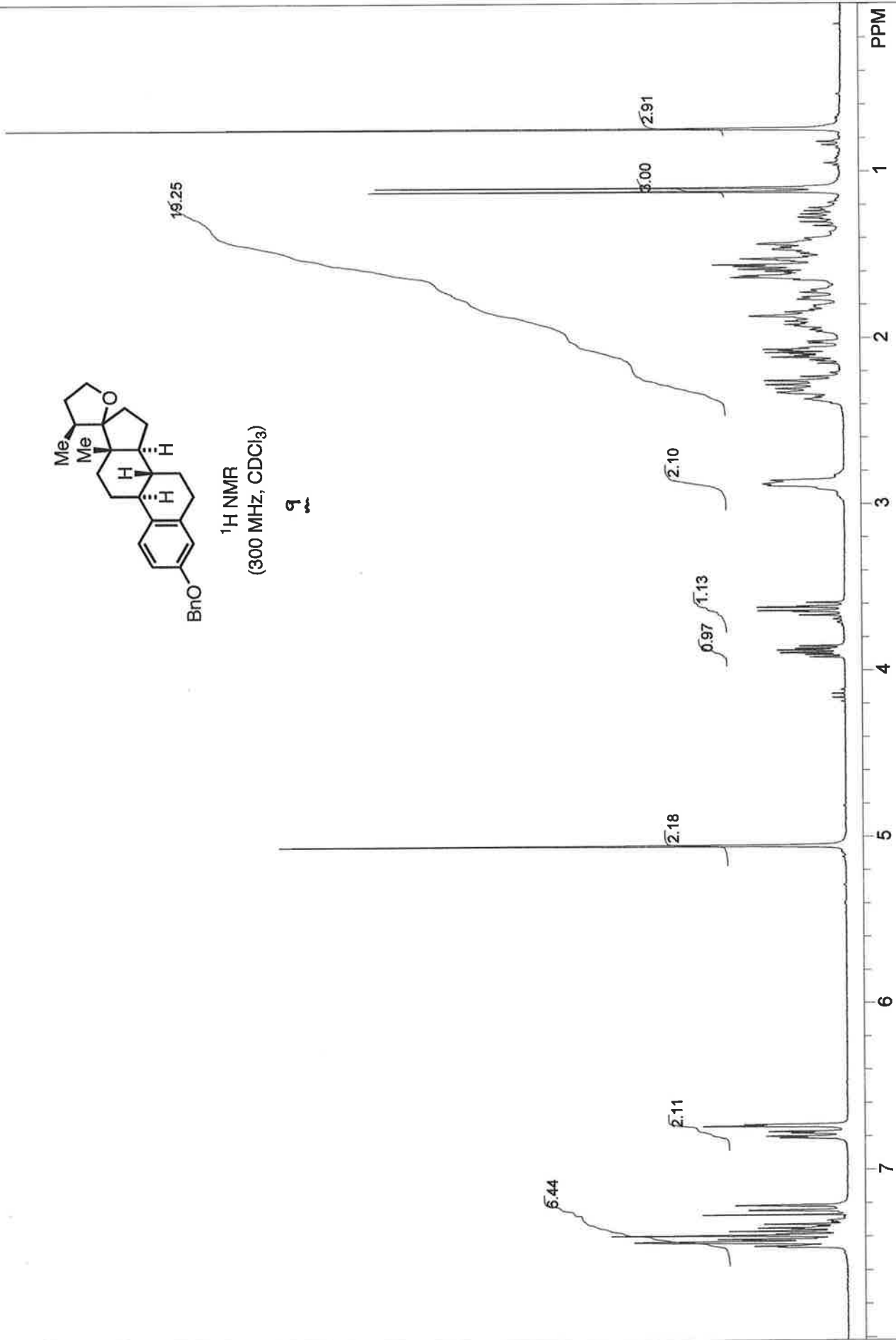


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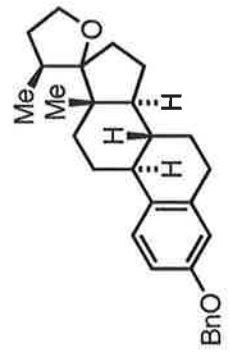
¹H NMR
(300 MHz, CDCl₃)

9



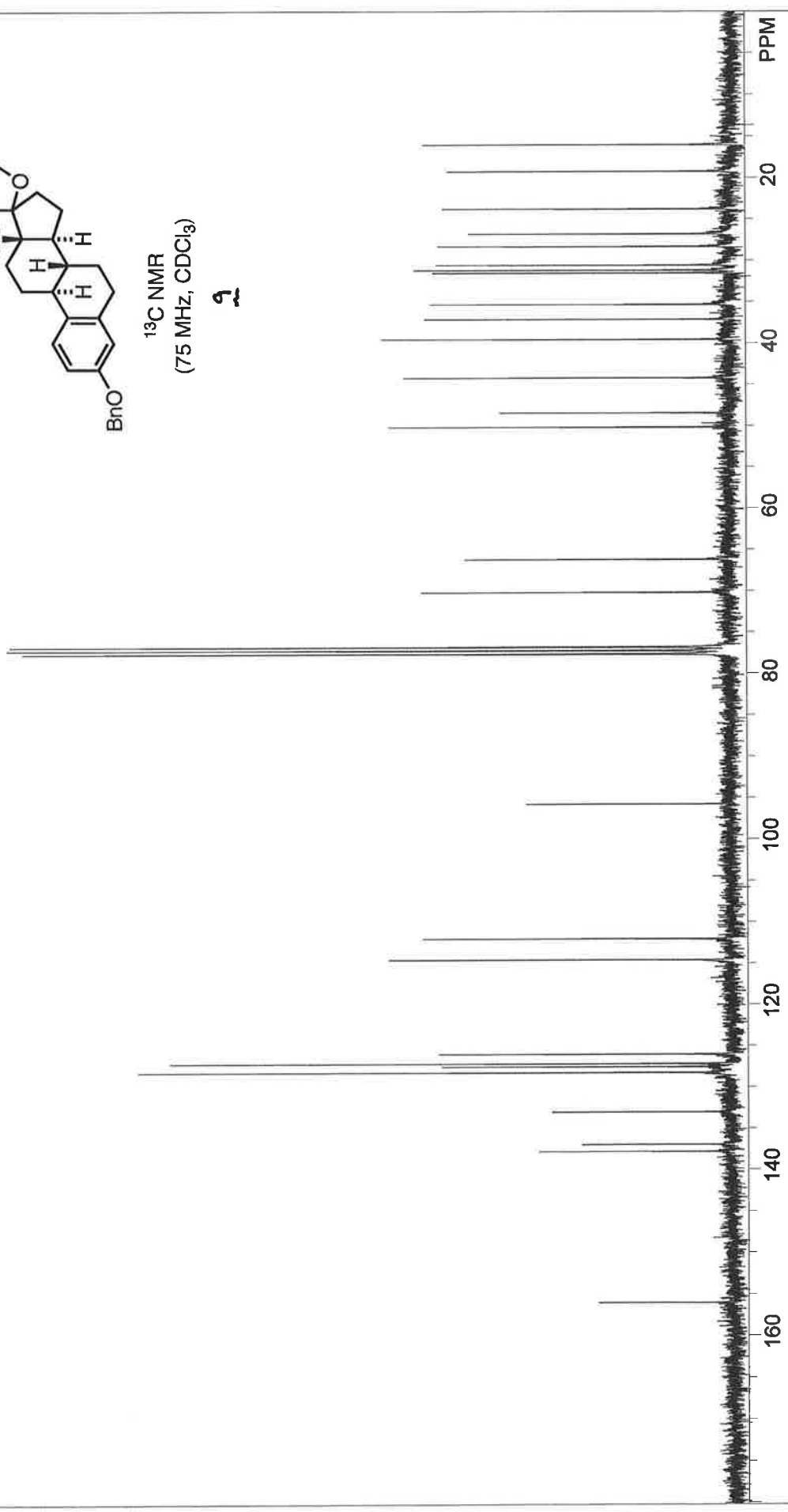
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16.043

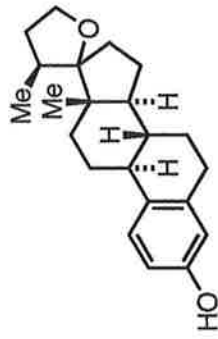


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2

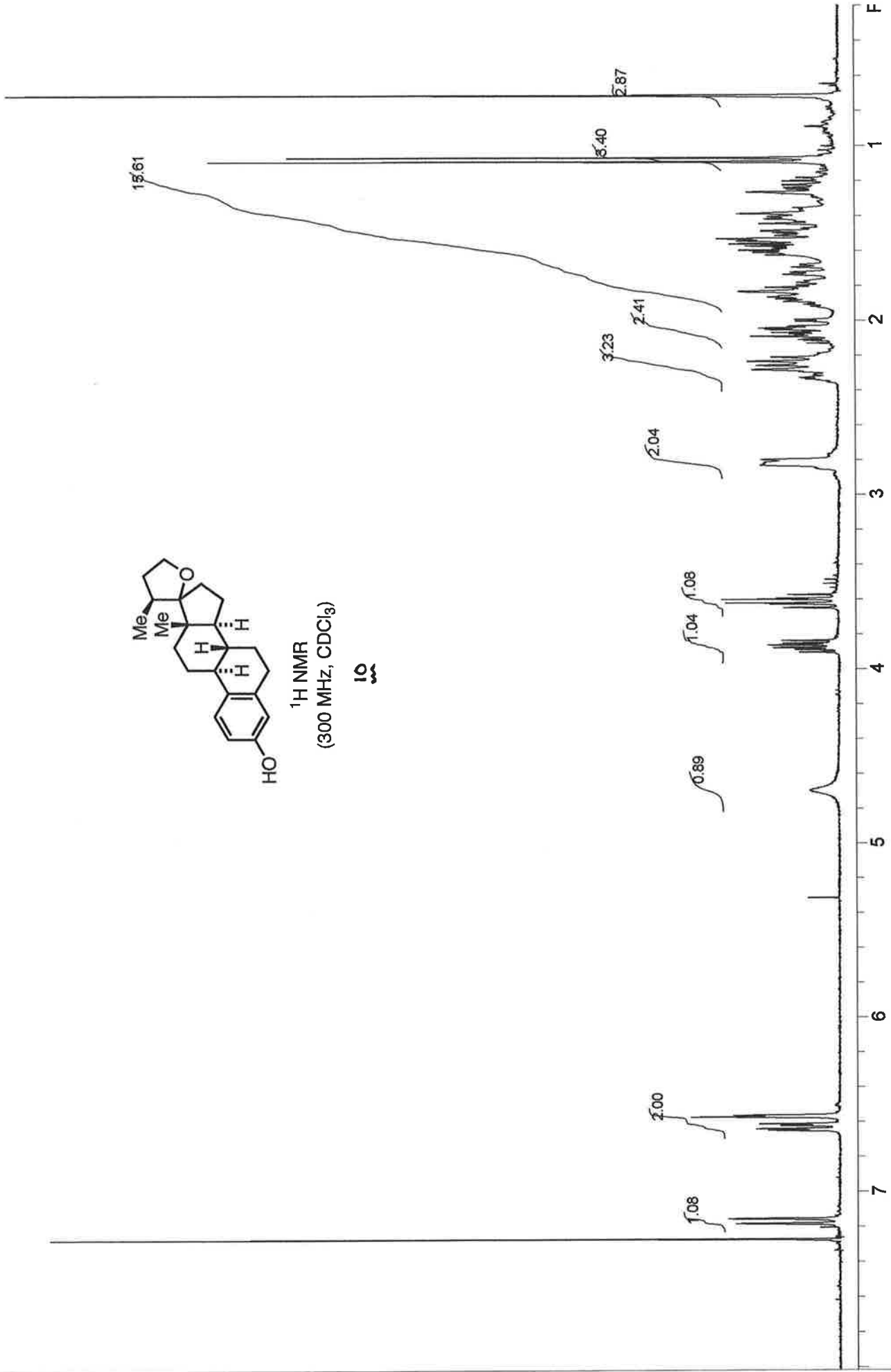


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¹H NMR
(300 MHz, CDCl₃)

10



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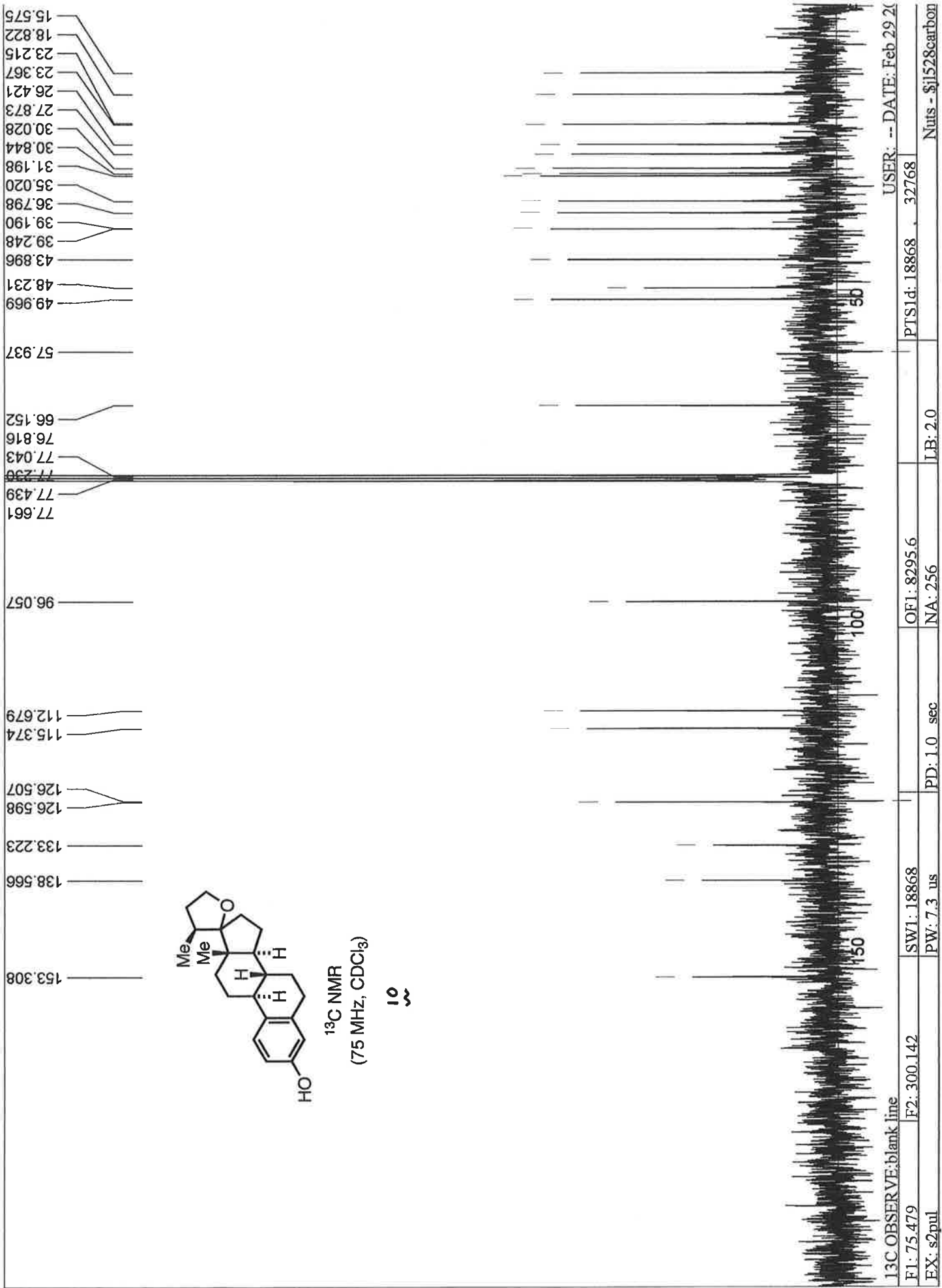
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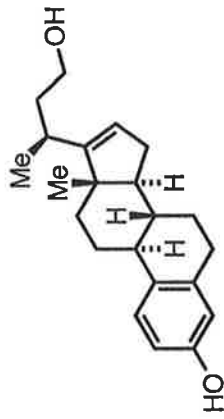
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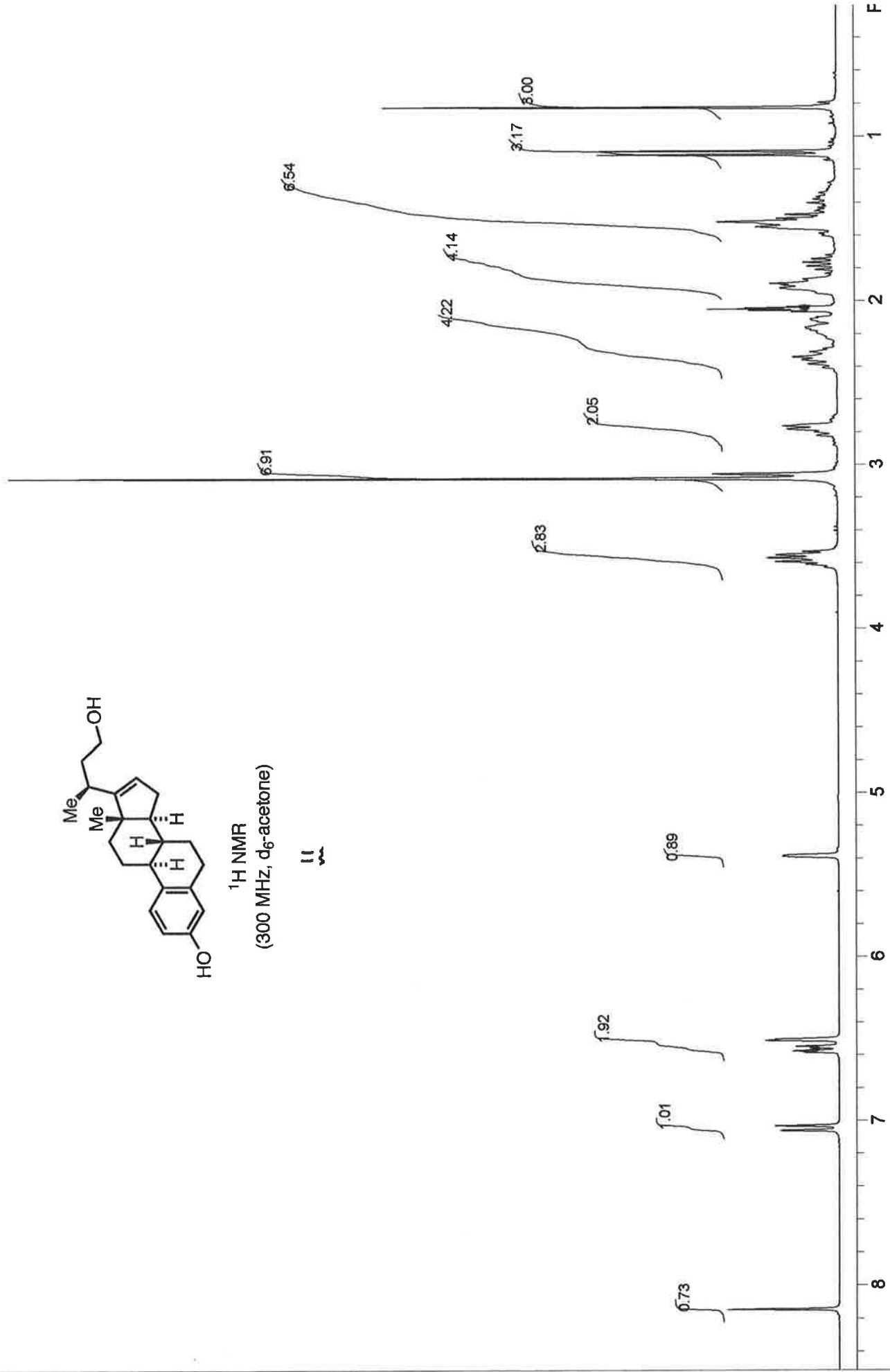
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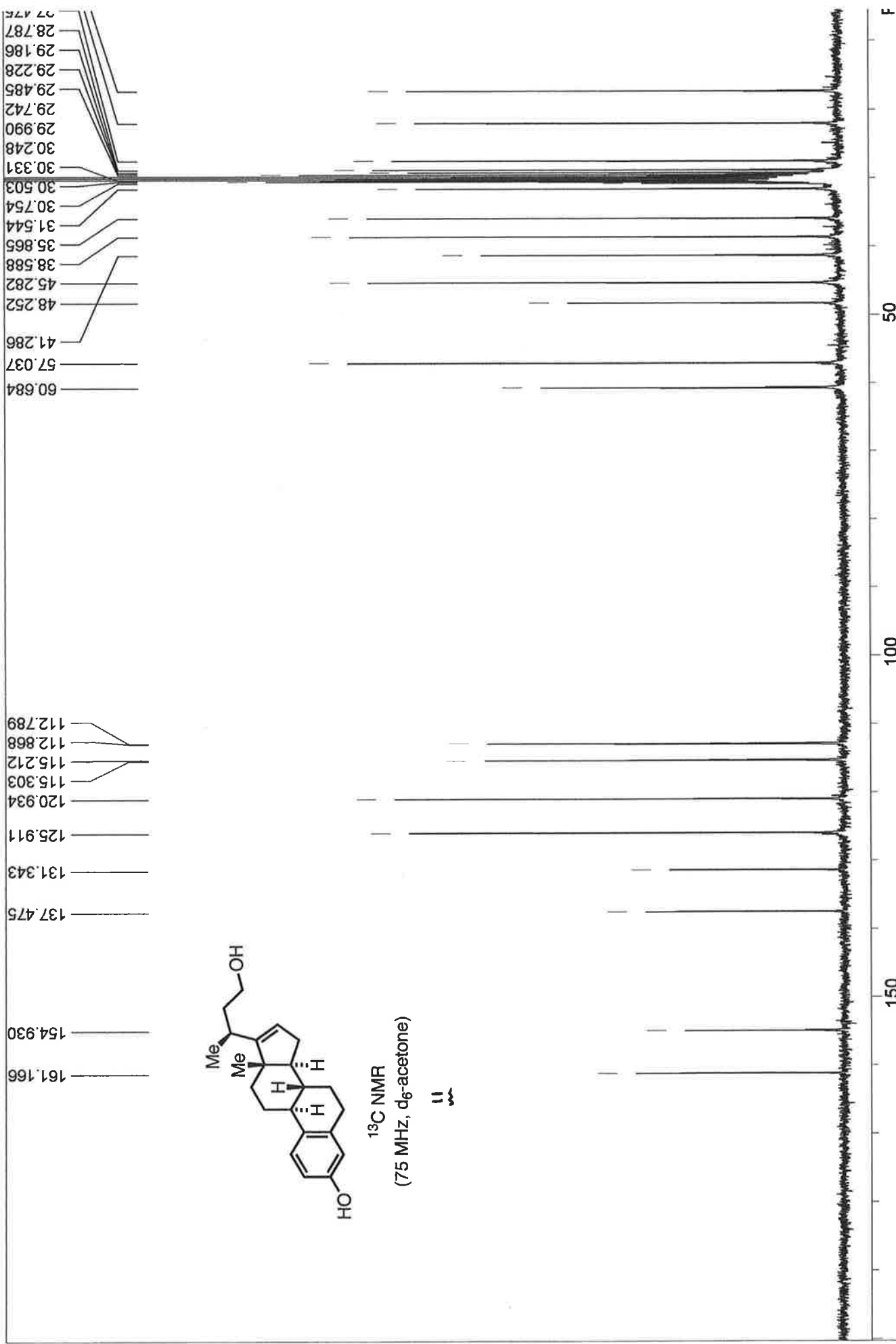


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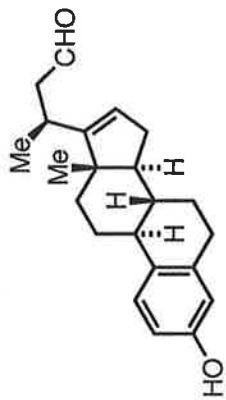
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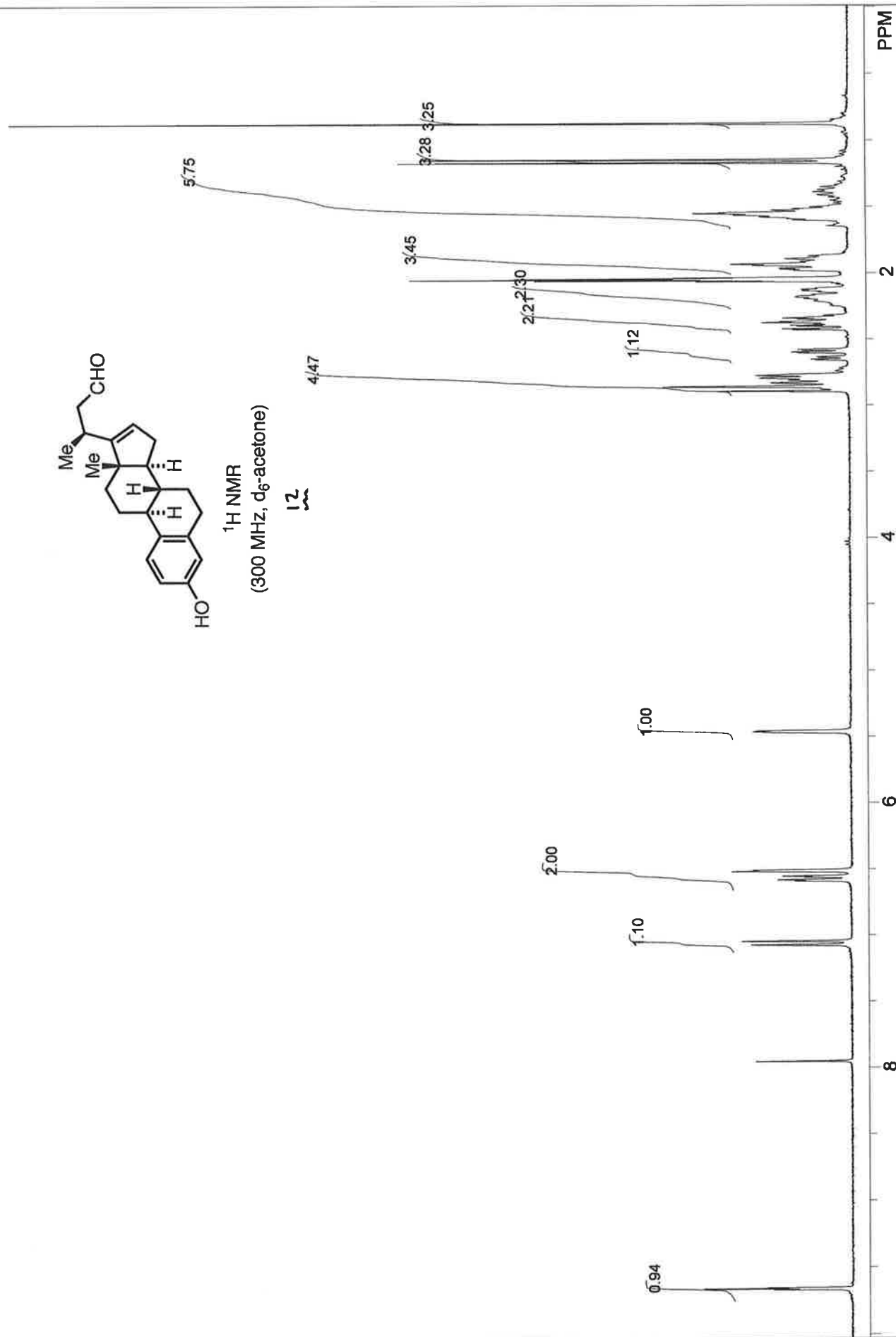


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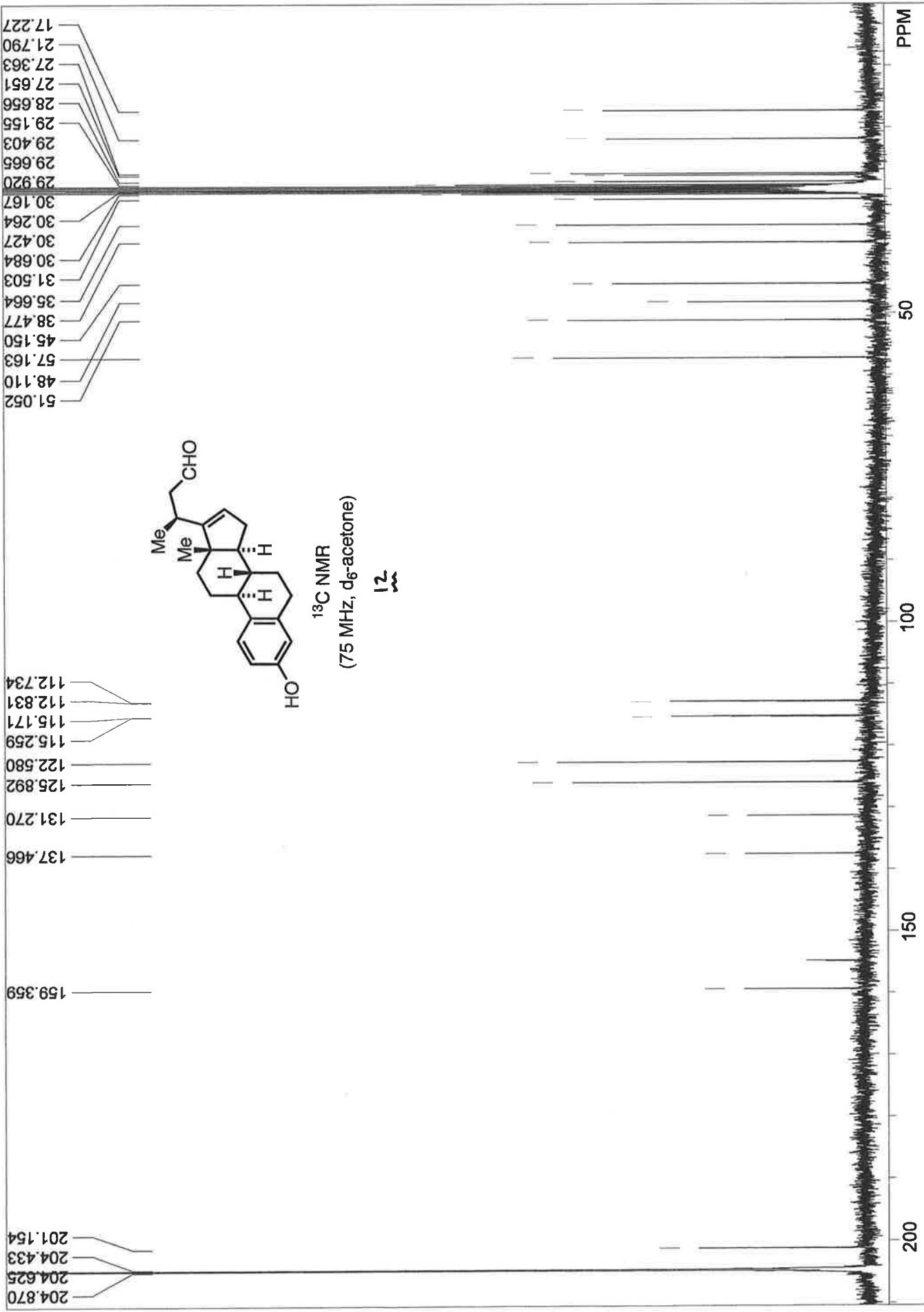


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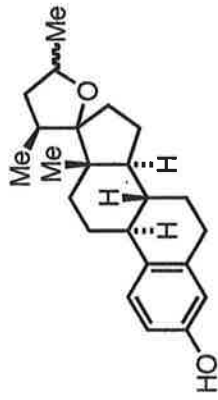
12



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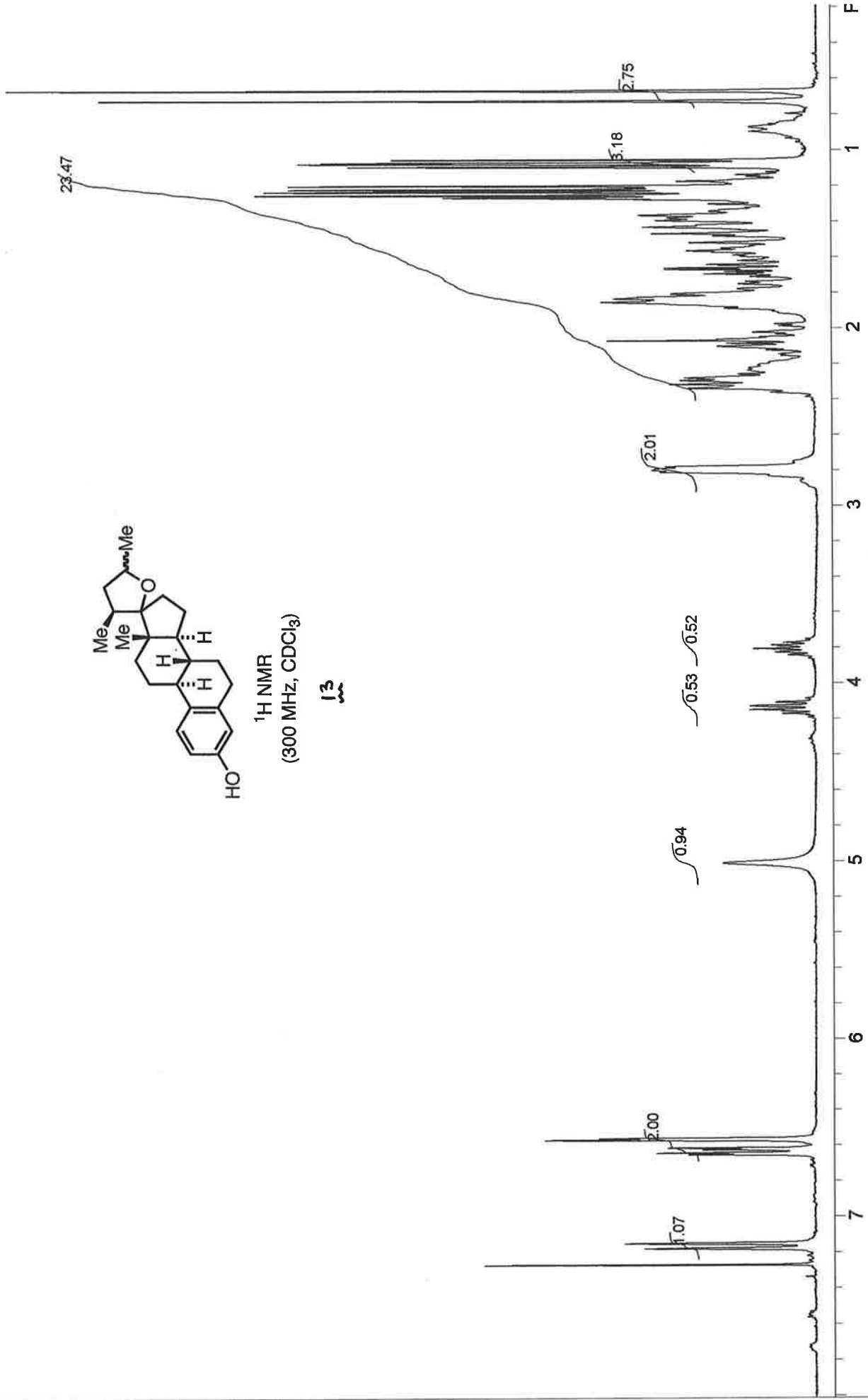


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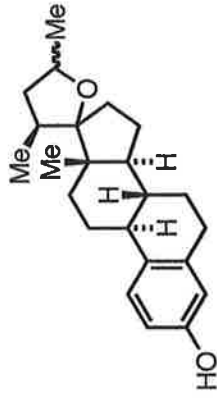


¹H NMR
(300 MHz, CDCl₃)

13

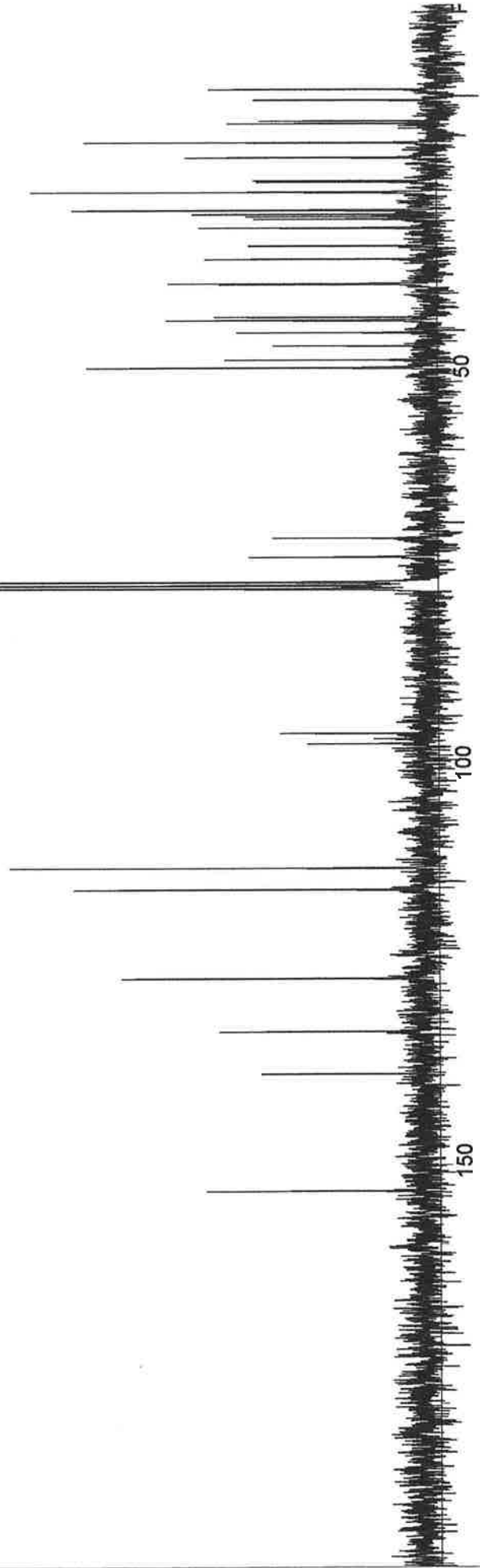


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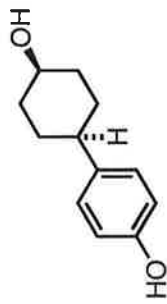


¹³C NMR
(75 MHz, CDCl₃)

13

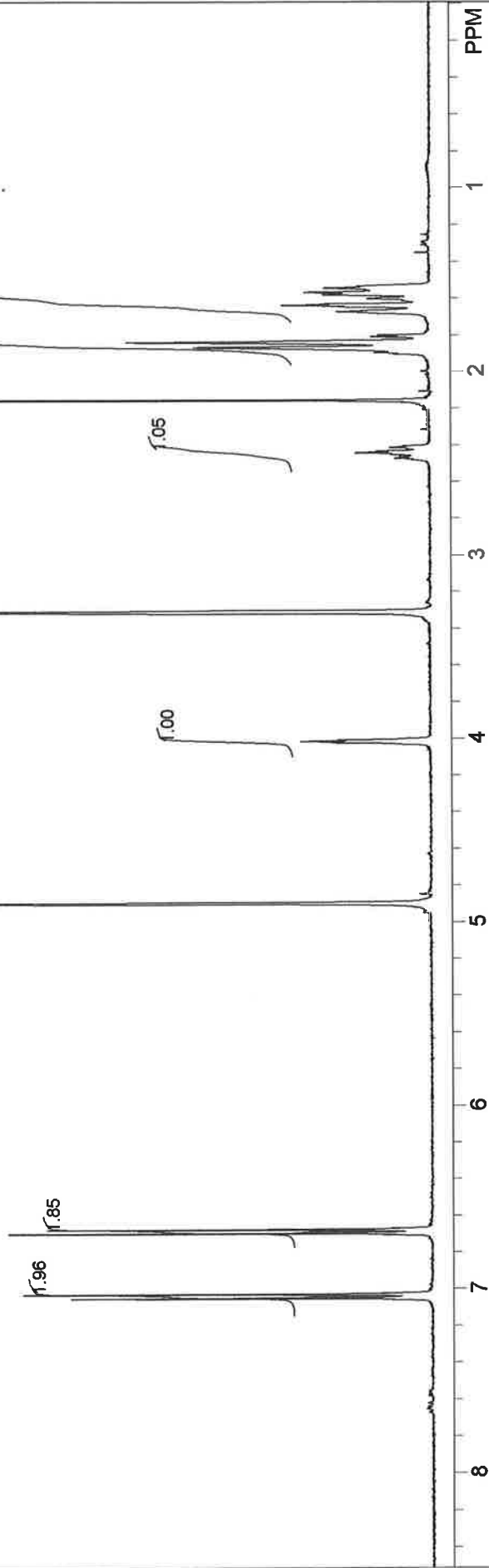


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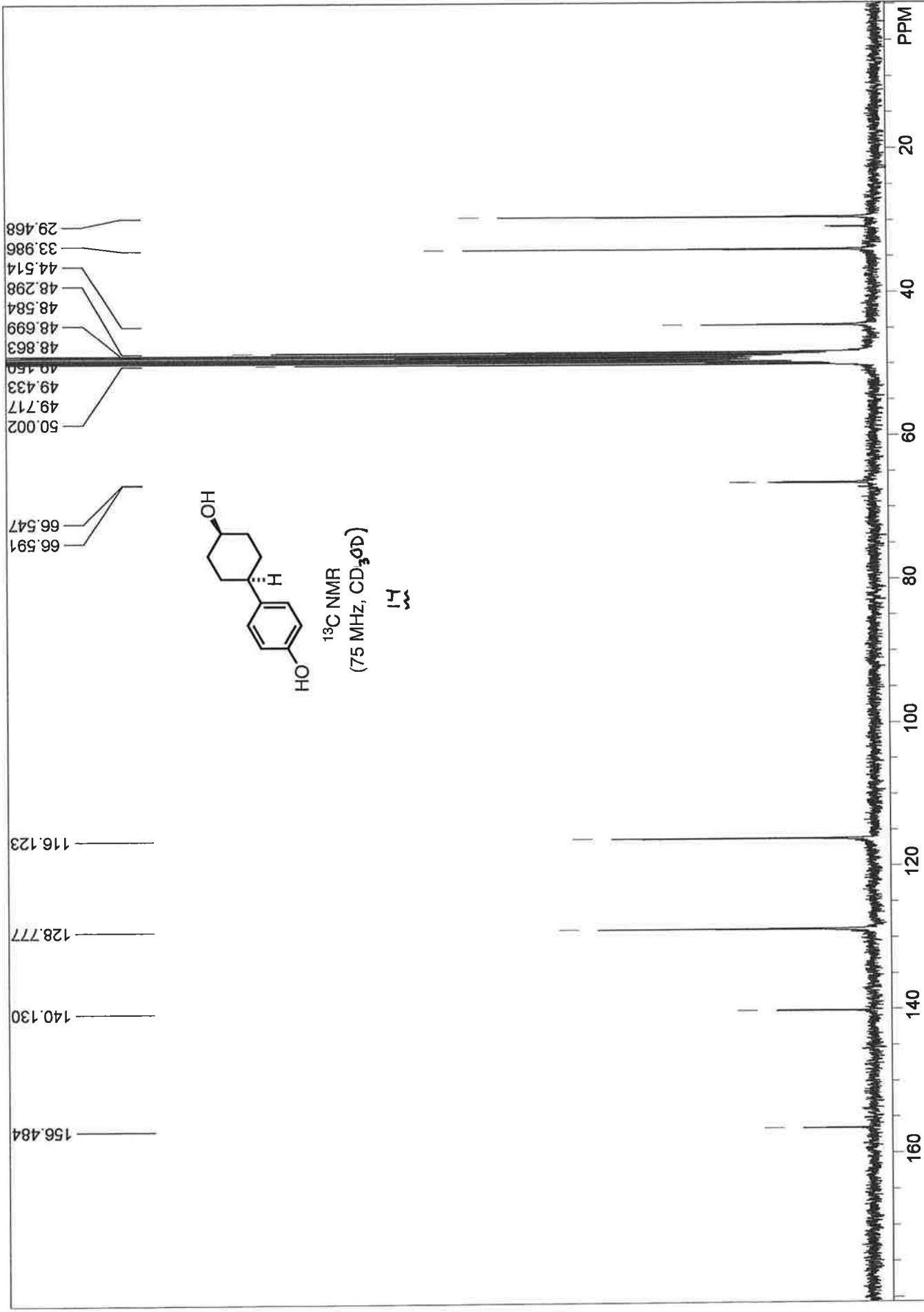
¹H NMR
(400 MHz, CD₃OD)

14

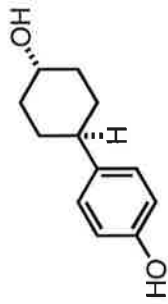


STANDARD 1H OBSERVE - profile

F1: 399.751	F2: 100.526	SW1: 6410	OF1: 827.0	PTS1d: 13132	16384	USER: -- DATE: Feb 19 2007
EX: s2pul		PW: 7.7 us	NA: 8	LB: 0.0		Nuts - \$rkpl30-[F-F-minor.fid

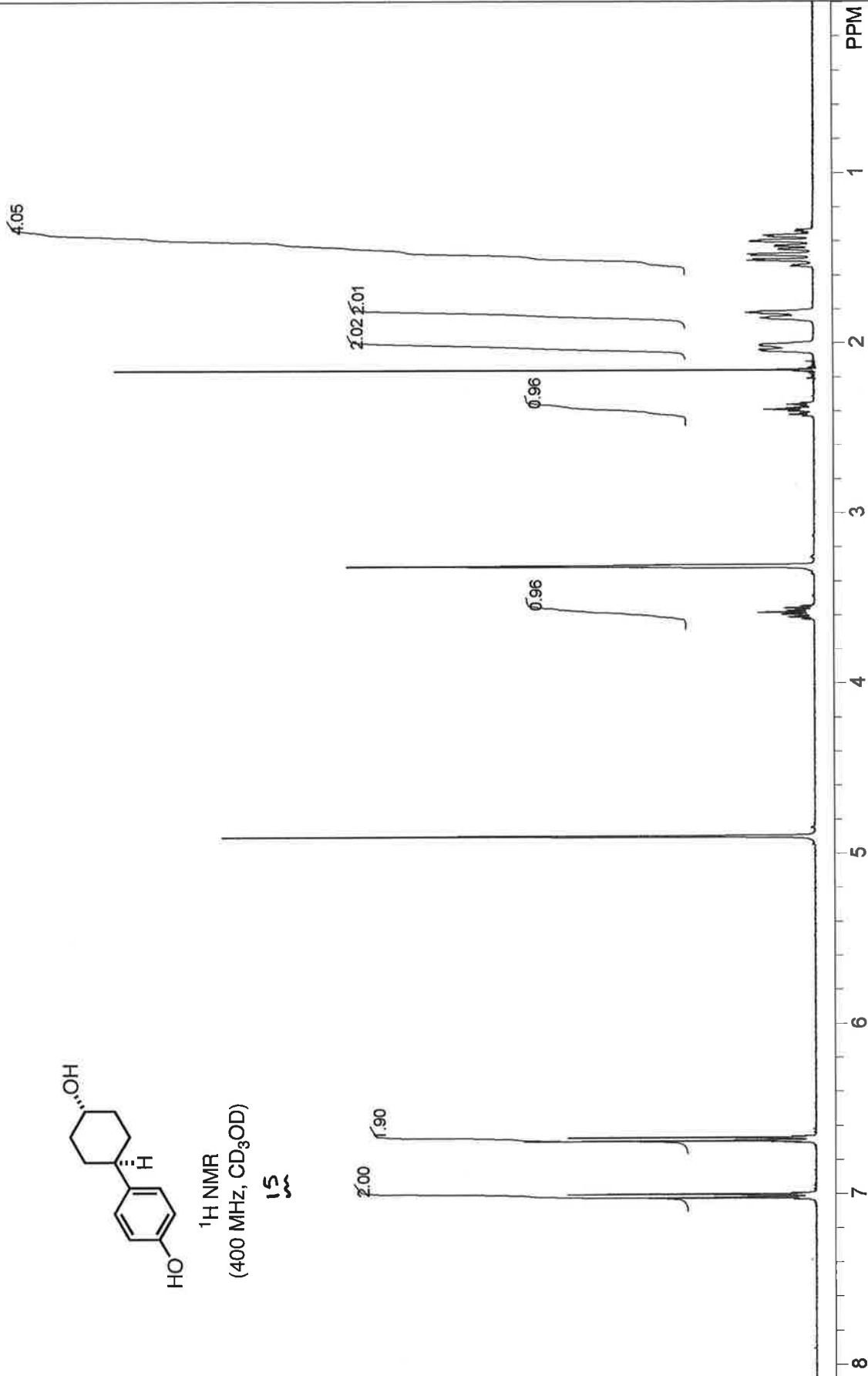


13C OBSERVE: blank line		USER: -- DATE: Feb 19 2007	
F1: 75.477	F2: 300.137	PTS1d: 34246	65536
EX: s2pul	SW1: 18868	NA: 64	LB: 1.5
	PW: 7.3 us	PD: 1.0 sec	OF1: 8402.5
			EX: s2pul



¹H NMR
(400 MHz, CD₃OD)

15



STANDARD 1H OBSERVE - profile

USER: -- DATE: Feb 19 2007

F1: 399.753 F2: 100.527 PTSId: 13132 16384

SW1: 6410 OF1: 2402.0

PW: 7.7 us PD: 1.0 sec

NA: 8

EX: s2pul

LB: 0.0

NA: 8

PD: 1.0 sec

PW: 7.7 us

PTSId: 13132 16384

USER: -- DATE: Feb 19 2007

EX: s2pul

LB: 0.0

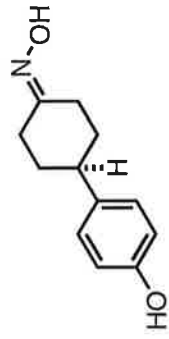
NA: 8

PD: 1.0 sec

PW: 7.7 us

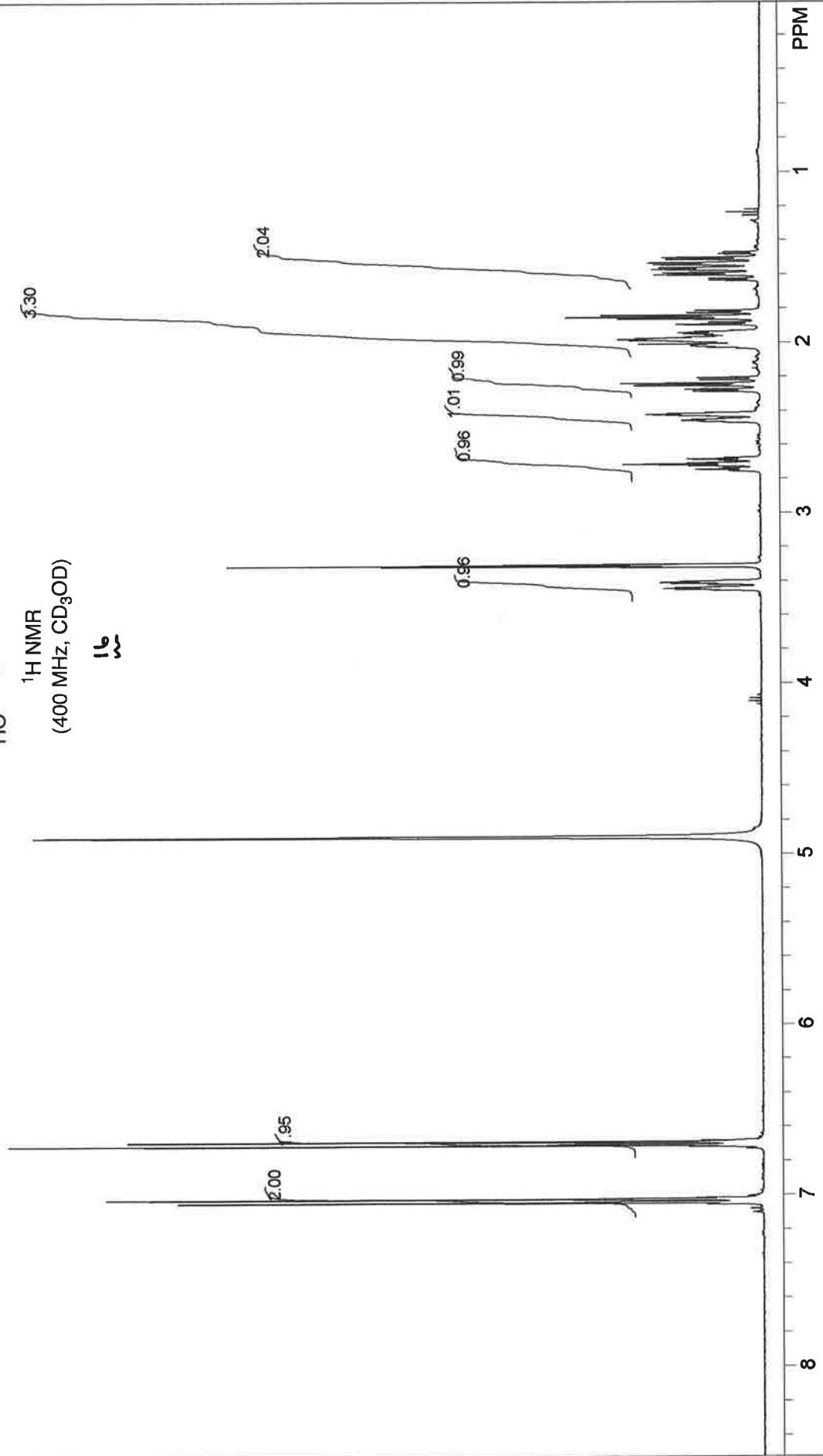
PTSId: 13132 16384

USER: -- DATE: Feb 19 2007



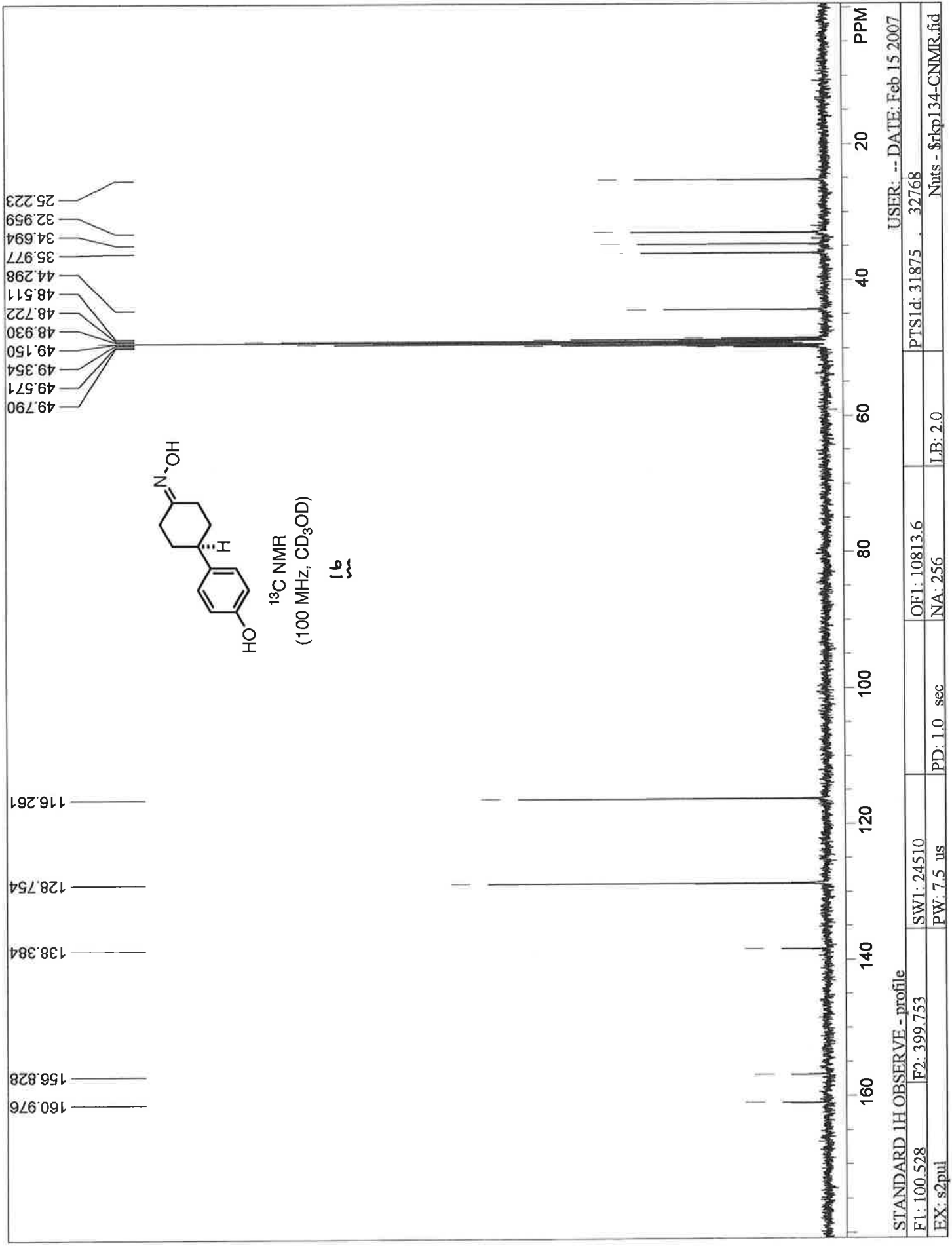
¹H NMR
(400 MHz, CD₃OD)

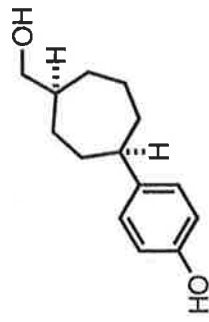
1b



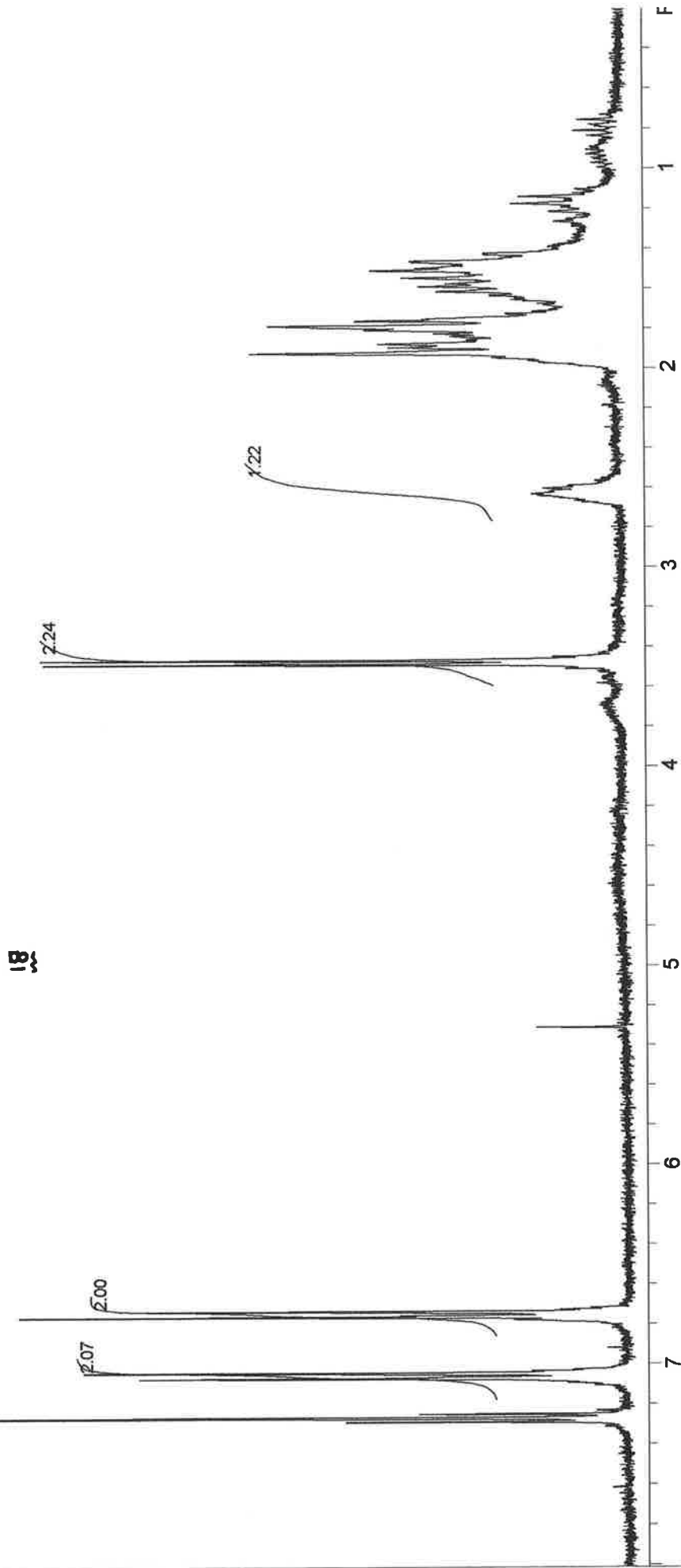
STANDARD 1H OBSERVE - profile

F1: 399.753	F2: 100.527	SW1: 6410	OF1: 2901.8	PTS1d: 13132	USER: -- DATE: Feb 15 2007
EX: s2pul	PW: 7.7 us	PD: 1.0 sec	NA: 8	LB: 0.0	16384
					Nuts - \$rkp134-crude.fid

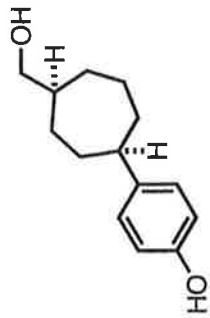




¹H NMR
(300 MHz, CDCl₃)



STANDARD 1H OBSERVE: blank line		USER: -- DATE: Oct 11 20	
F1: 300.145	F2: 75.479	PTS1d: 14430	16384
EX: s2pul	PD: 1.0 sec	LB: 0.0	Nuts - \$rkp086-II-F-HINMR
OF1: 1804.1	NA: 64		



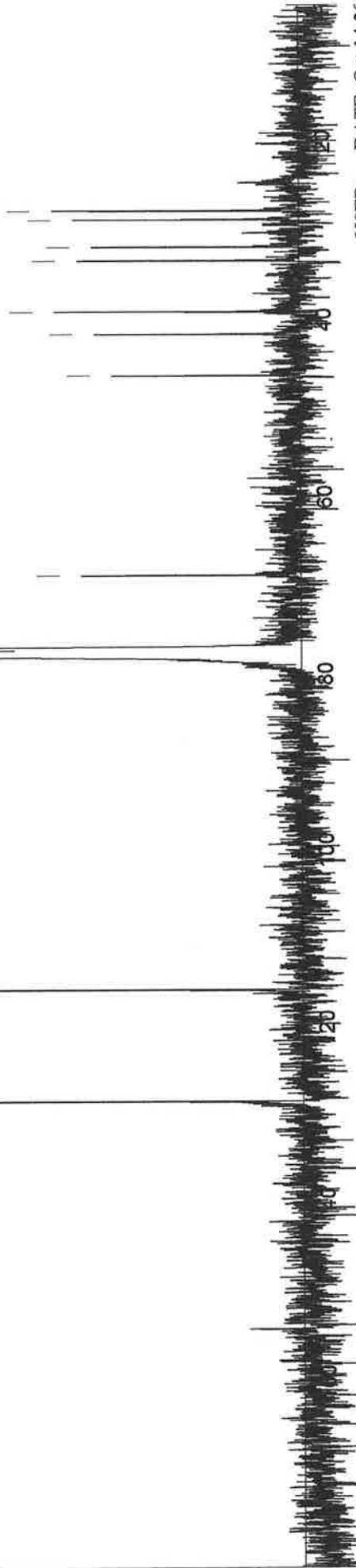
¹³C NMR
(75 MHz, CDCl₃)

18

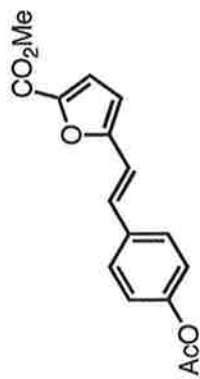
46.050
41.391
38.846
33.113
31.577
28.528
27.526

77.649
77.418
77.230
76.802
68.587

127.888
115.277

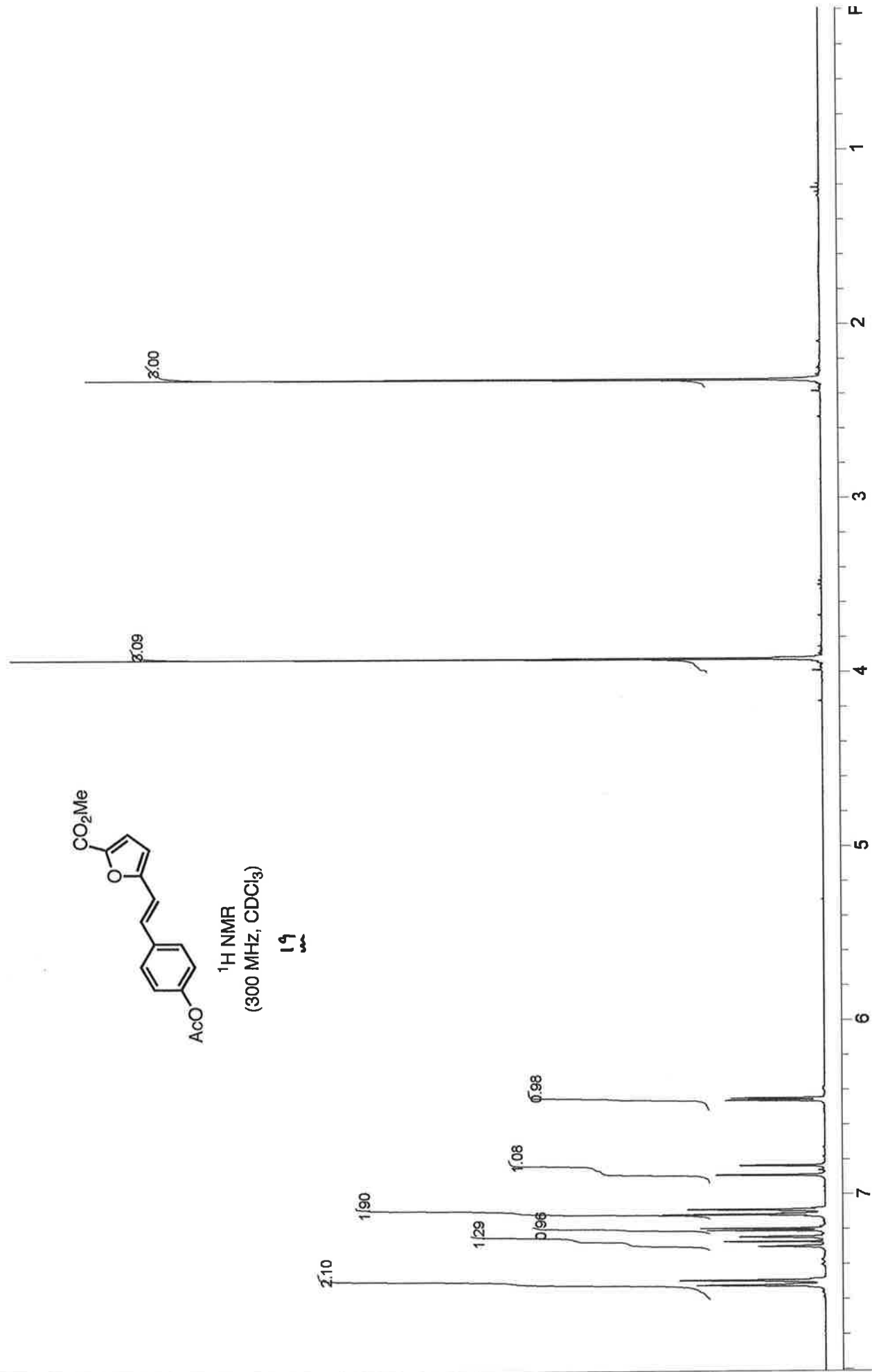


13C OBSERVE: blank line		USER: -- DATE: Oct 14 20	
F1: 75.479	F2: 300.145	PTS1d: 18868	32768
EX: s2pul		LB: 2.0	
		NA: 24000	
		OF1: 8300.1	
		PD: 1.0 sec	
		SW1: 18868	
		PW: 7.3 us	
			Nuts - \$tkp086-II-F-CNMNR

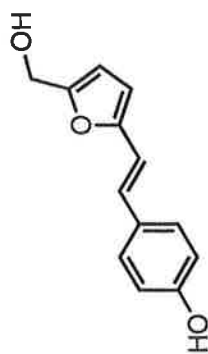


¹H NMR
(300 MHz, CDCl₃)

19

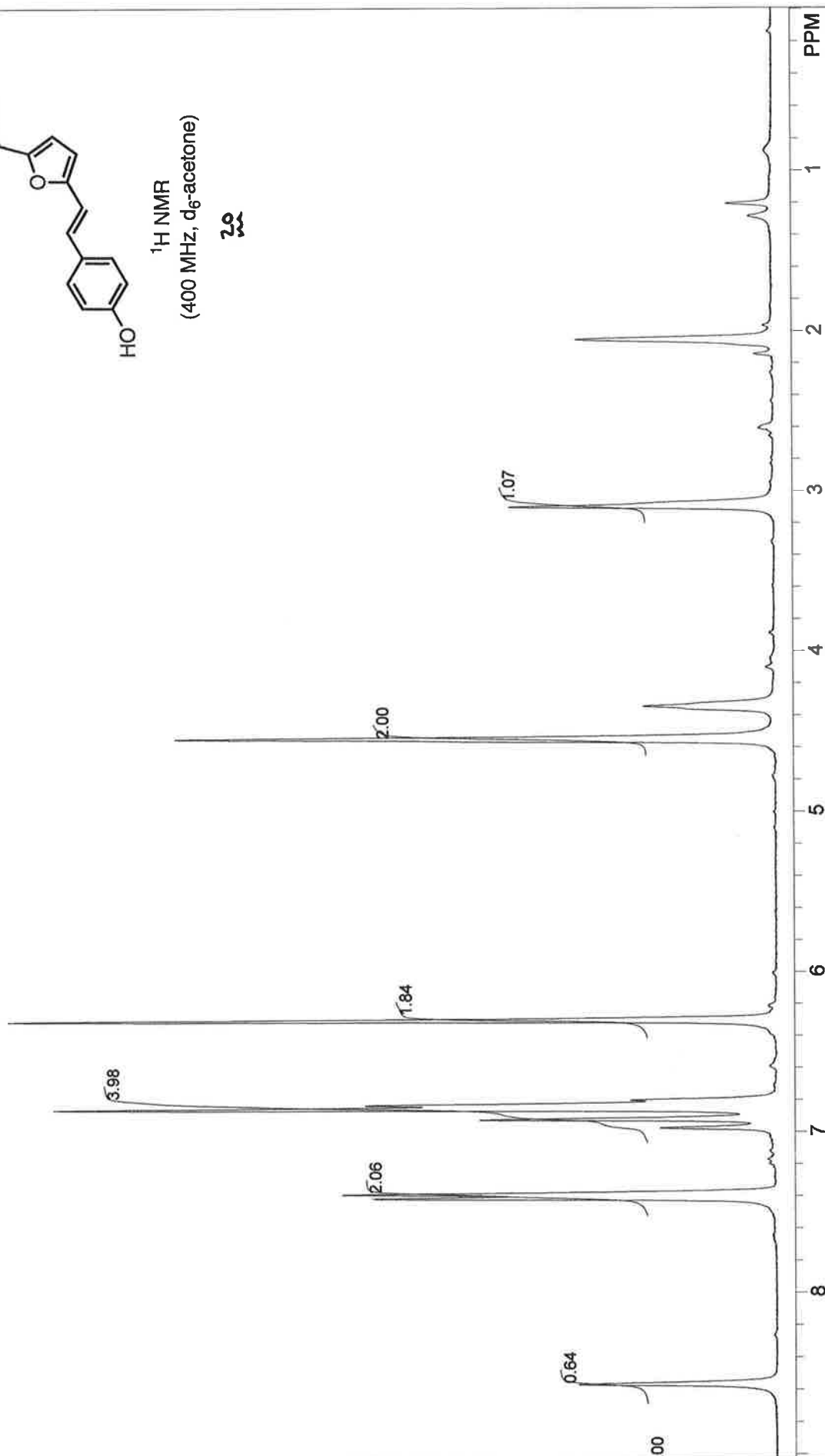


STANDARD 1H OBSERVE:blank line		USER: -- DATE: Sep 21 20	
F1: 300.145	F2: 75.479	PTS1d: 14409	16384
EX: s2pul	PD: 1.0 sec	LB: 0.0	Nuts - \$rkp-HH-sample

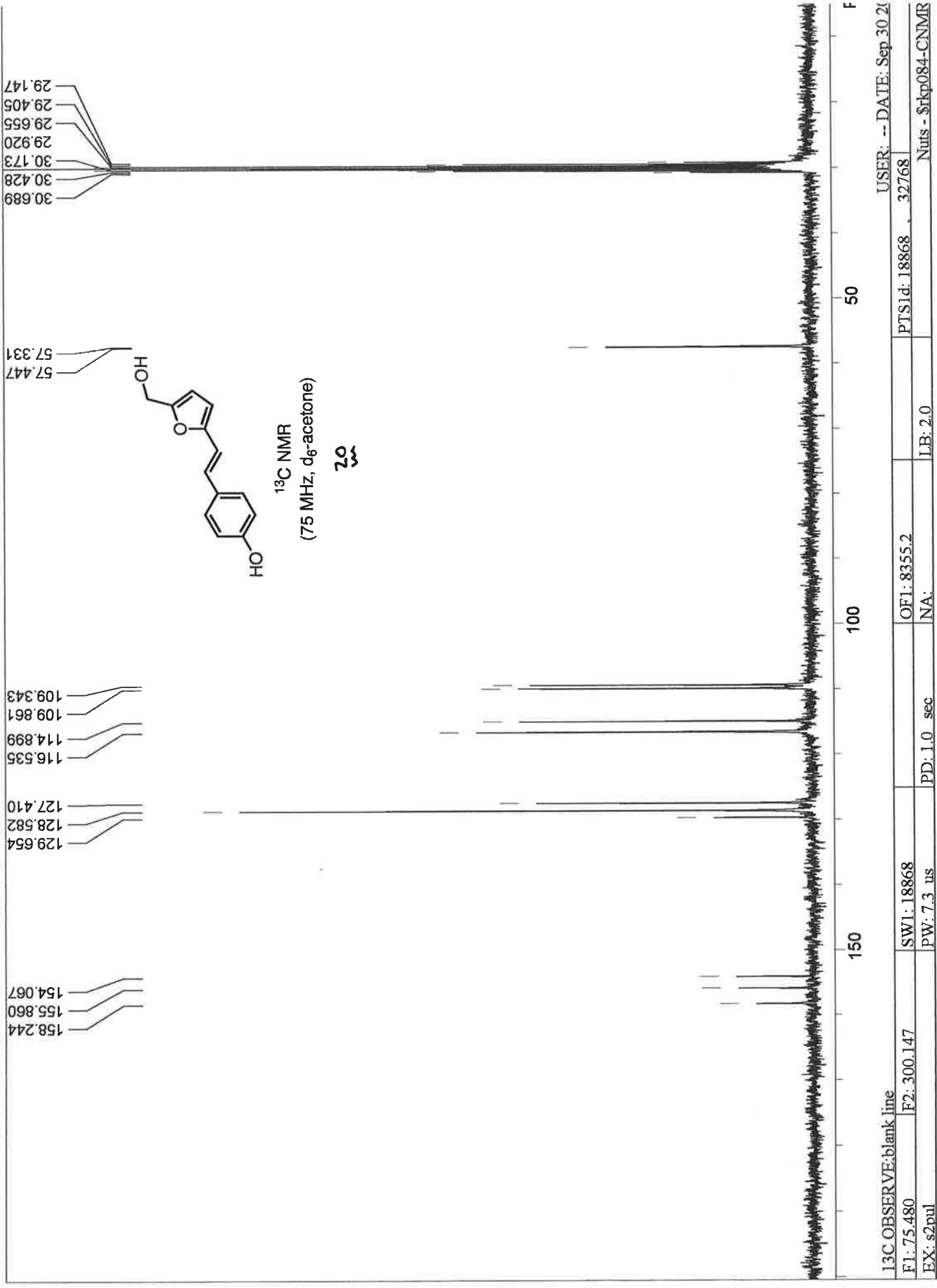


¹H NMR
(400 MHz, d₆-acetone)

2.0



STANDARD 1H OBSERVE: blank line		USER: -- DATE: Sep 30 2006	
F1: 300.147	F2: 75.479	PTS1d: 14409	16384
EX: s2pul	PD: 1.0 sec	OF1: 1800.9	NA: 8
		LB: 0.0	Nuts - \$rkp084-HNMR



13C OBSERVE: blank line	USER: -- DATE: Sep 30 20
F1: 75.480	PTS1d: 18868 . 32768
F2: 300.147	OF1: 8355.2
EX: s2pul	NA:
	PD: 1.0 sec
	PW: 7.3 us
	SW1: 18868
	LB: 2.0
	Nuts - \$rkp084-CNMIR