

Supplementary Information

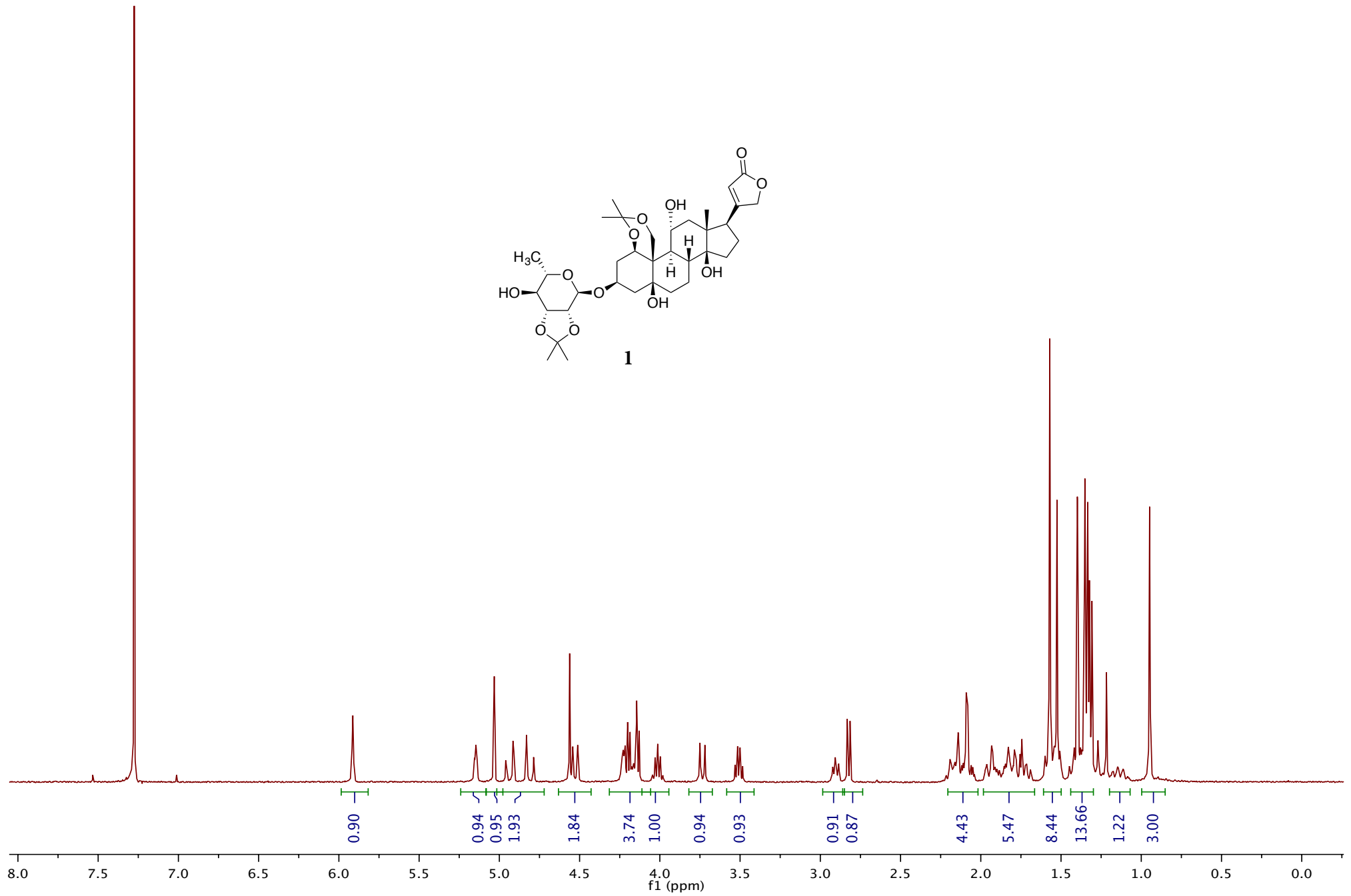
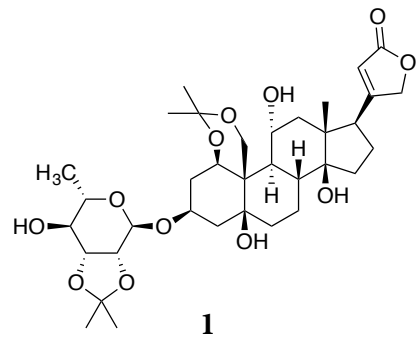
Design, Synthesis, in vitro and in vivo Evaluation of Ouabain Analogs as Potent and Selective Na,K-ATPase $\alpha 4$ Isoform Inhibitors for Male Contraception

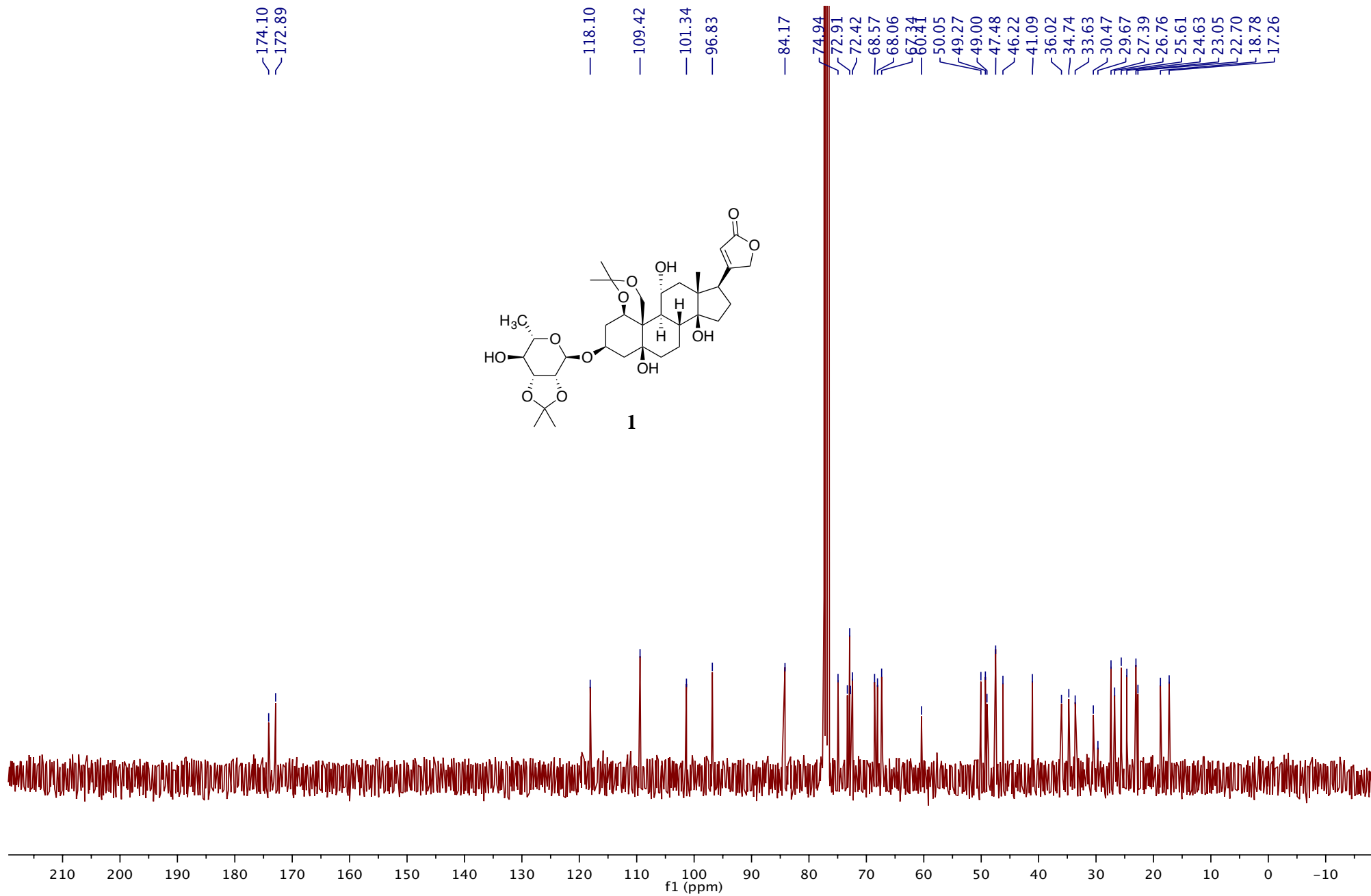
*Shameem Sultana Syeda,¹ Gladis Sánchez,² Kwon Ho Hong,¹
Jon E. Hawkinson,¹ Gunda I. Georg,^{1*} and Gustavo Blanco^{2*}*

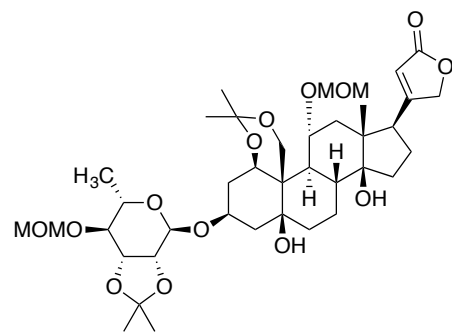
¹Department of Medicinal Chemistry and Institute for Therapeutics Discovery and Development,
College of Pharmacy, University of Minnesota, Minneapolis, MN 55414, USA

²Department of Molecular and Integrative Physiology, University of Kansas Medical Center,
Kansas City, KS 66160, USA

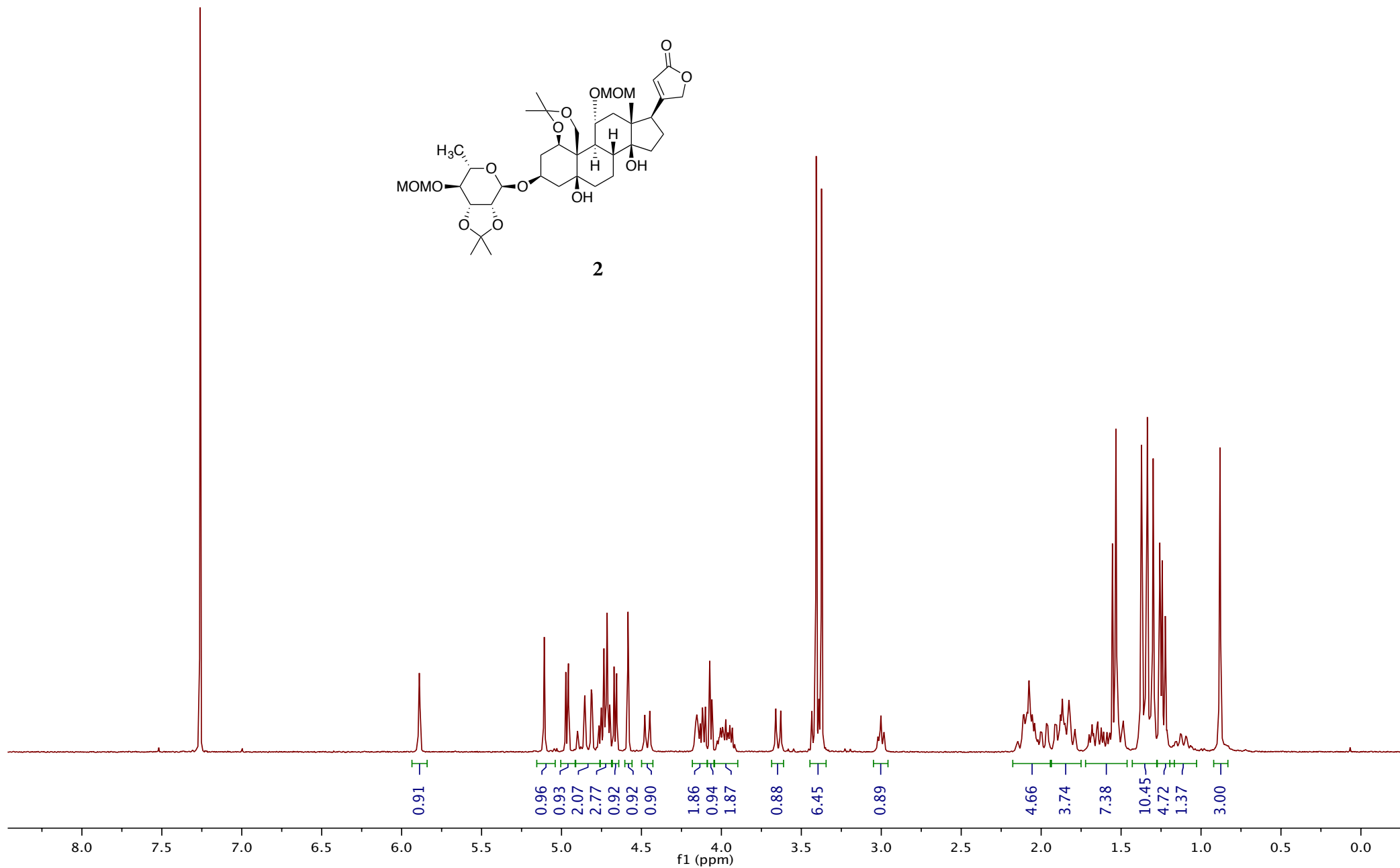
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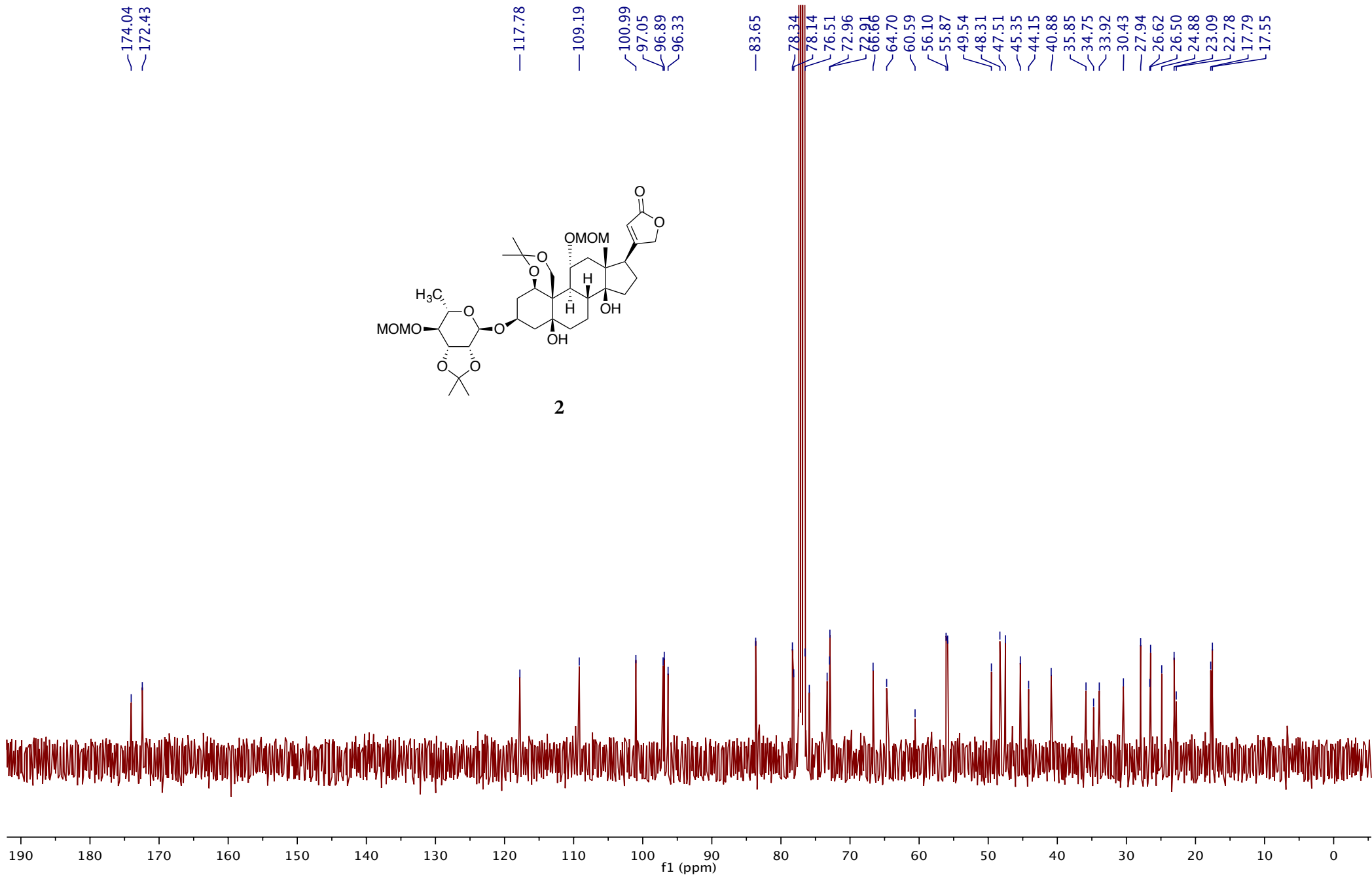
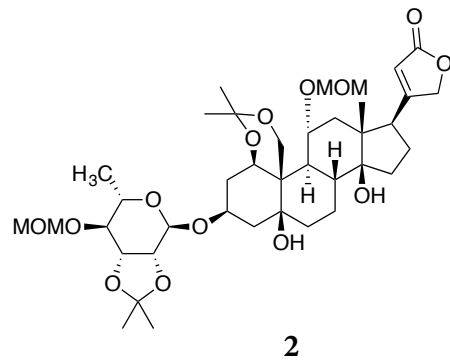


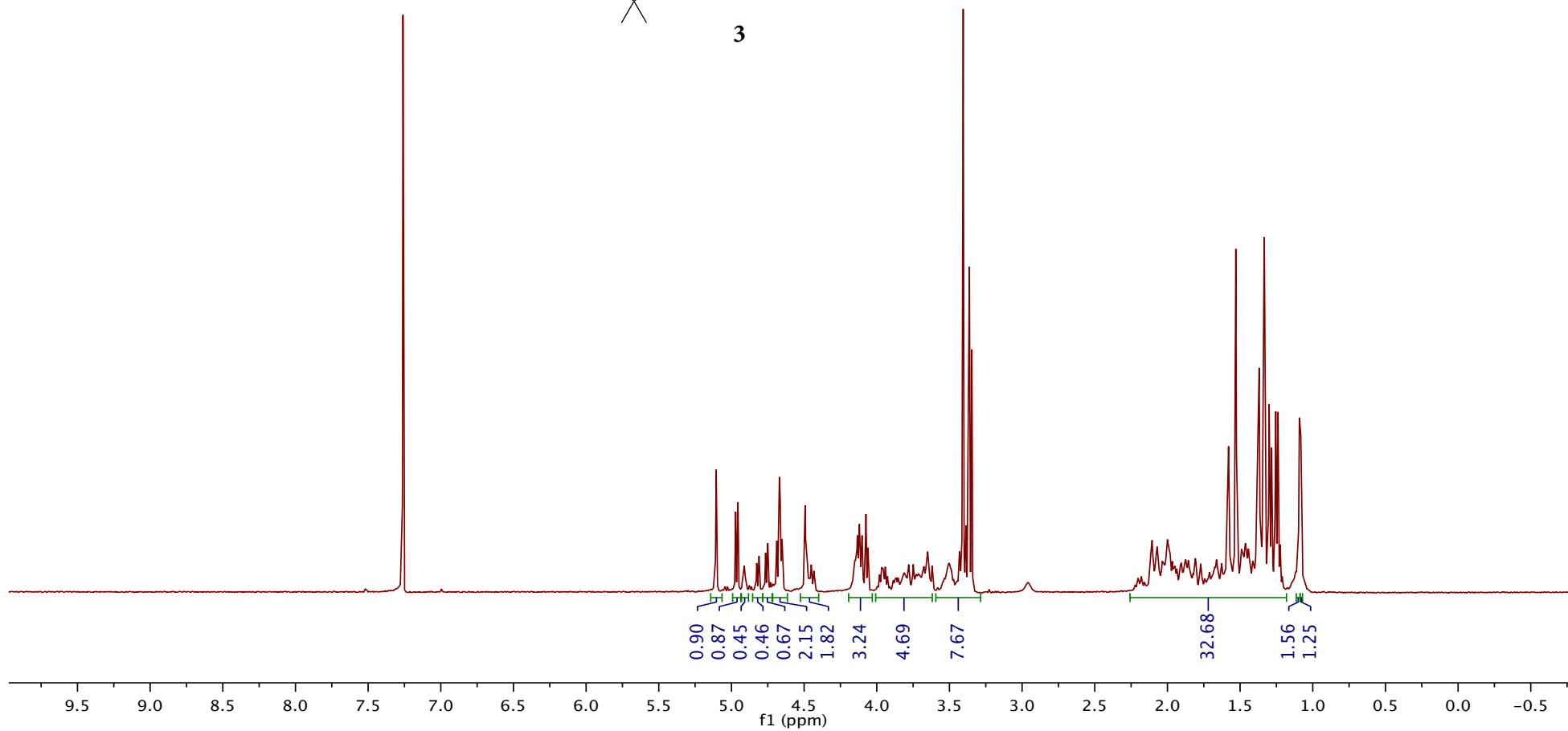
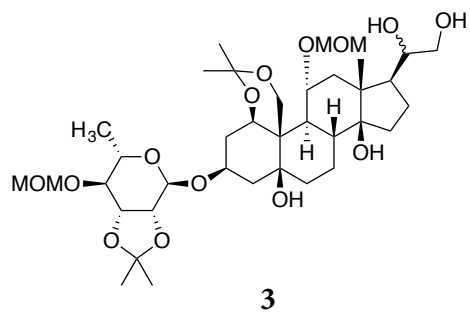


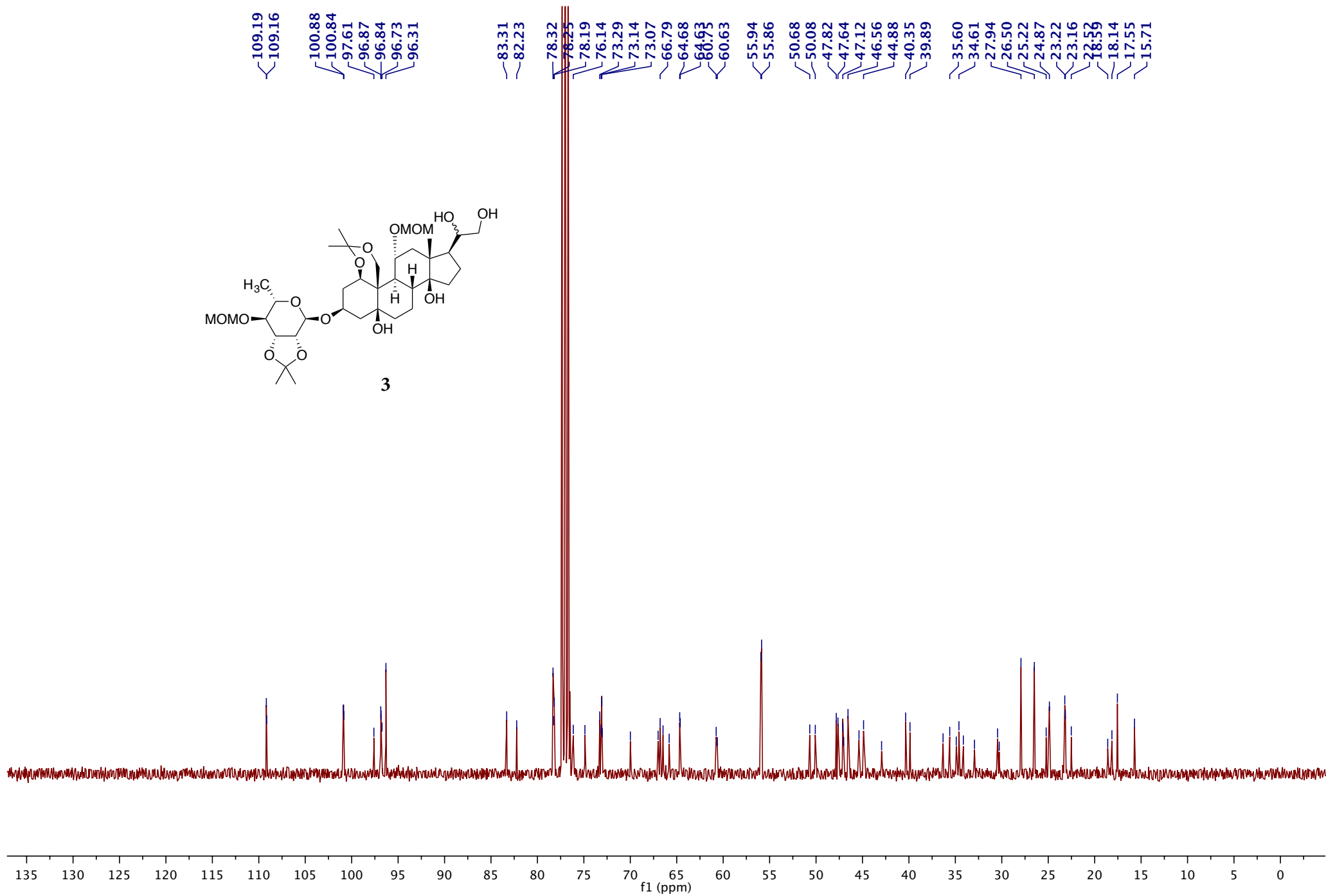
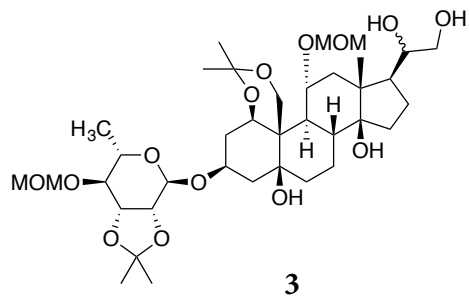


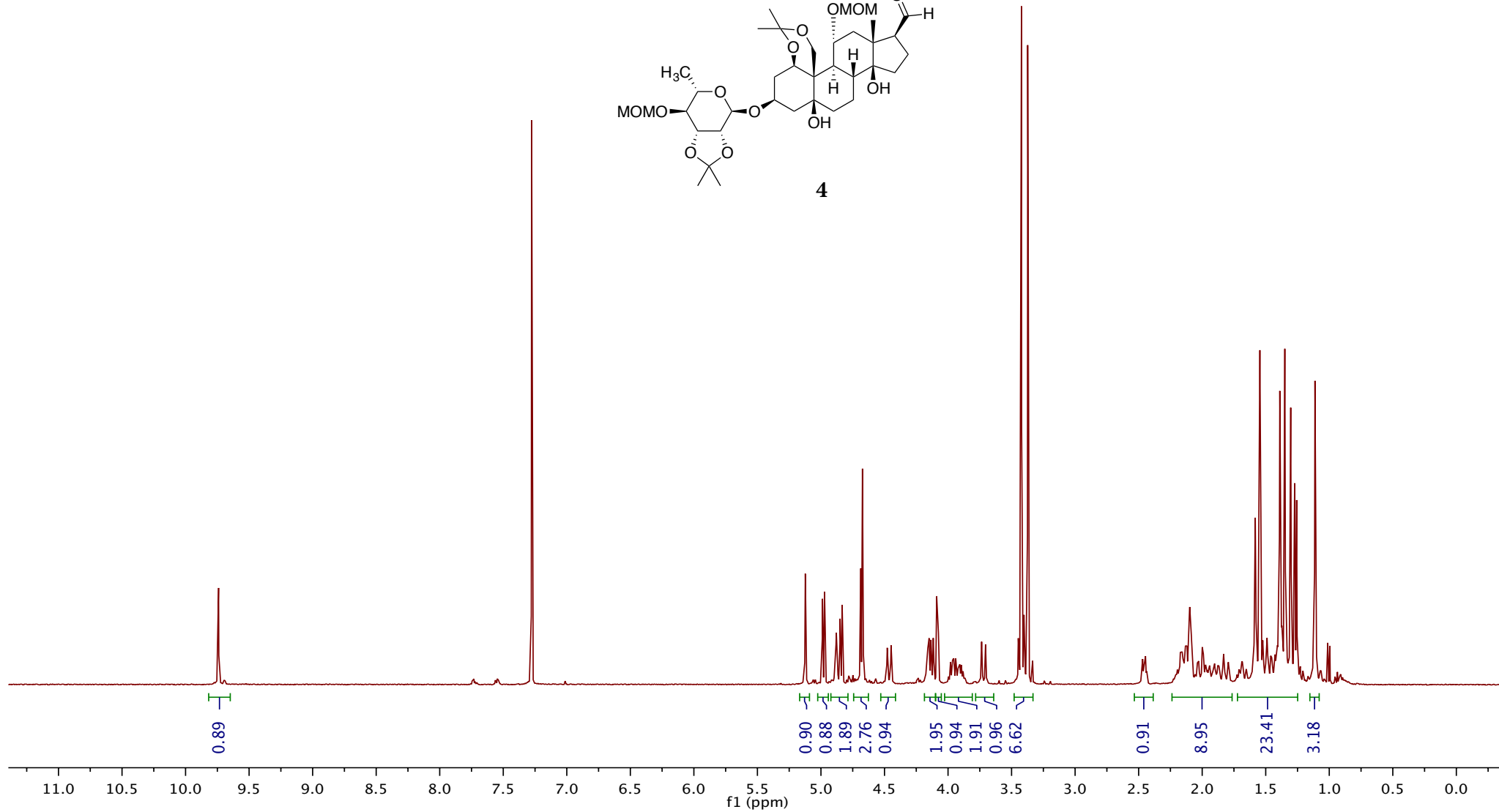
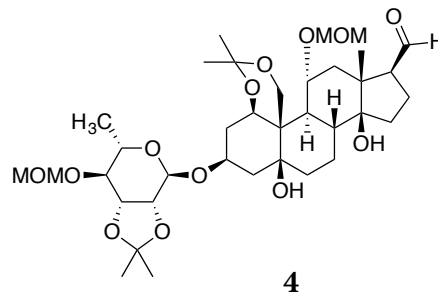
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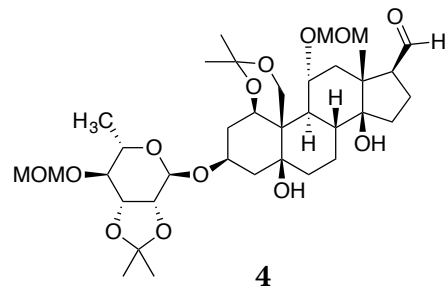




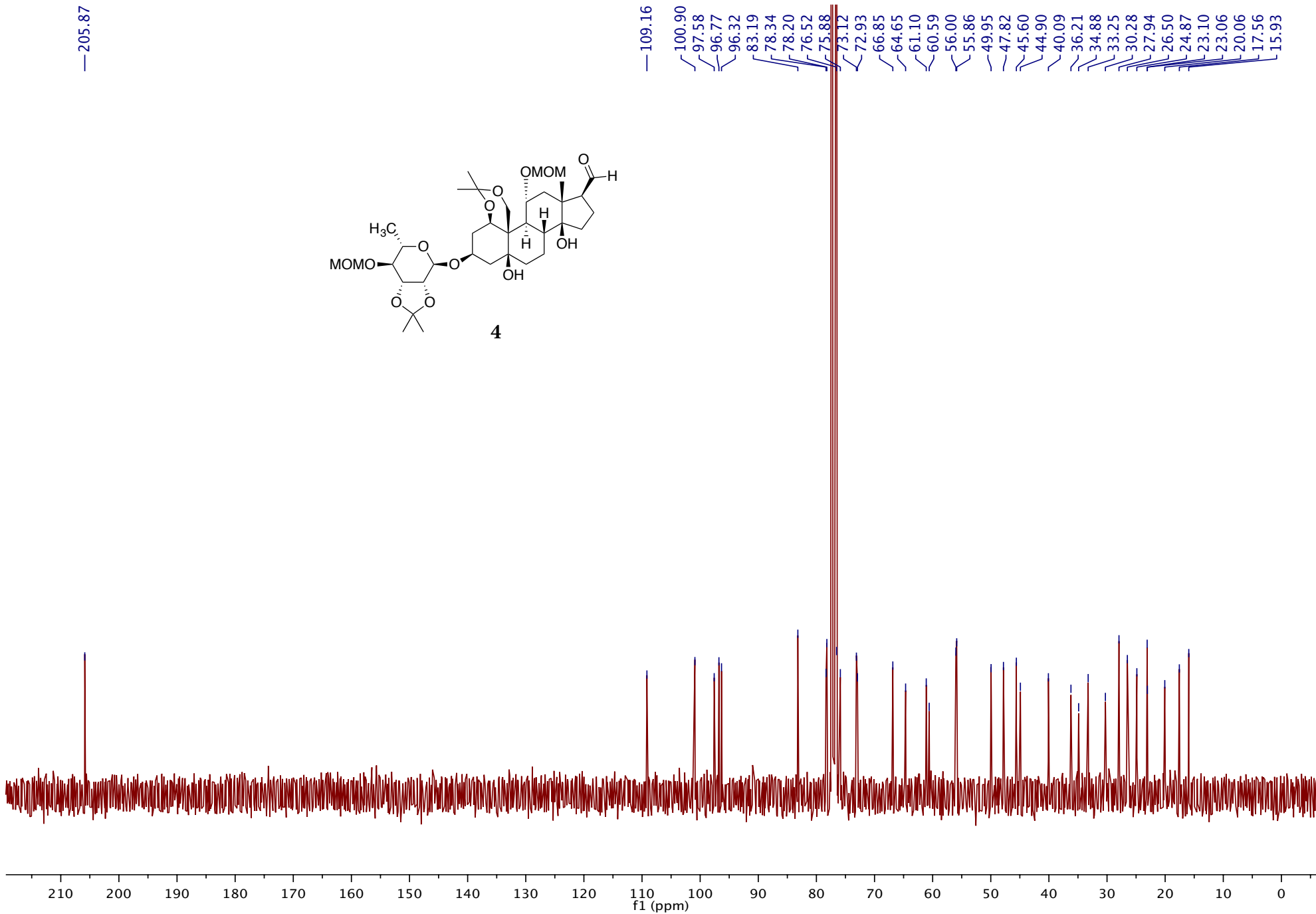


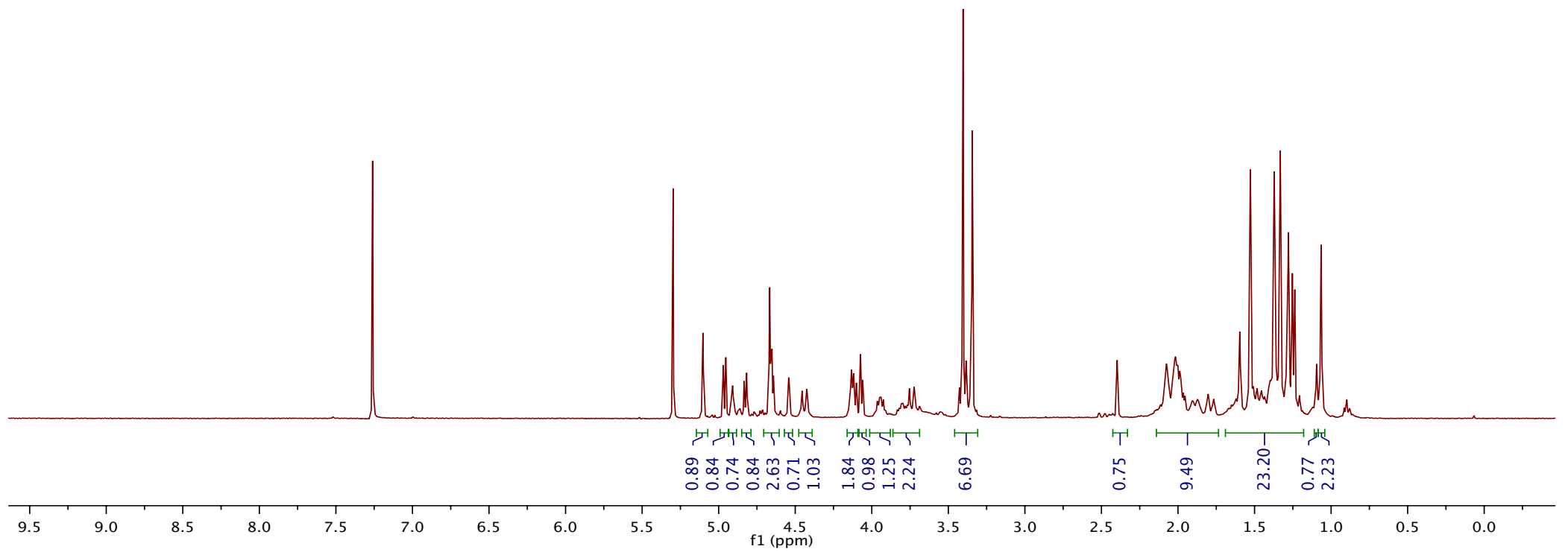
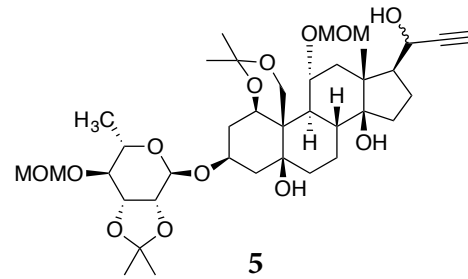


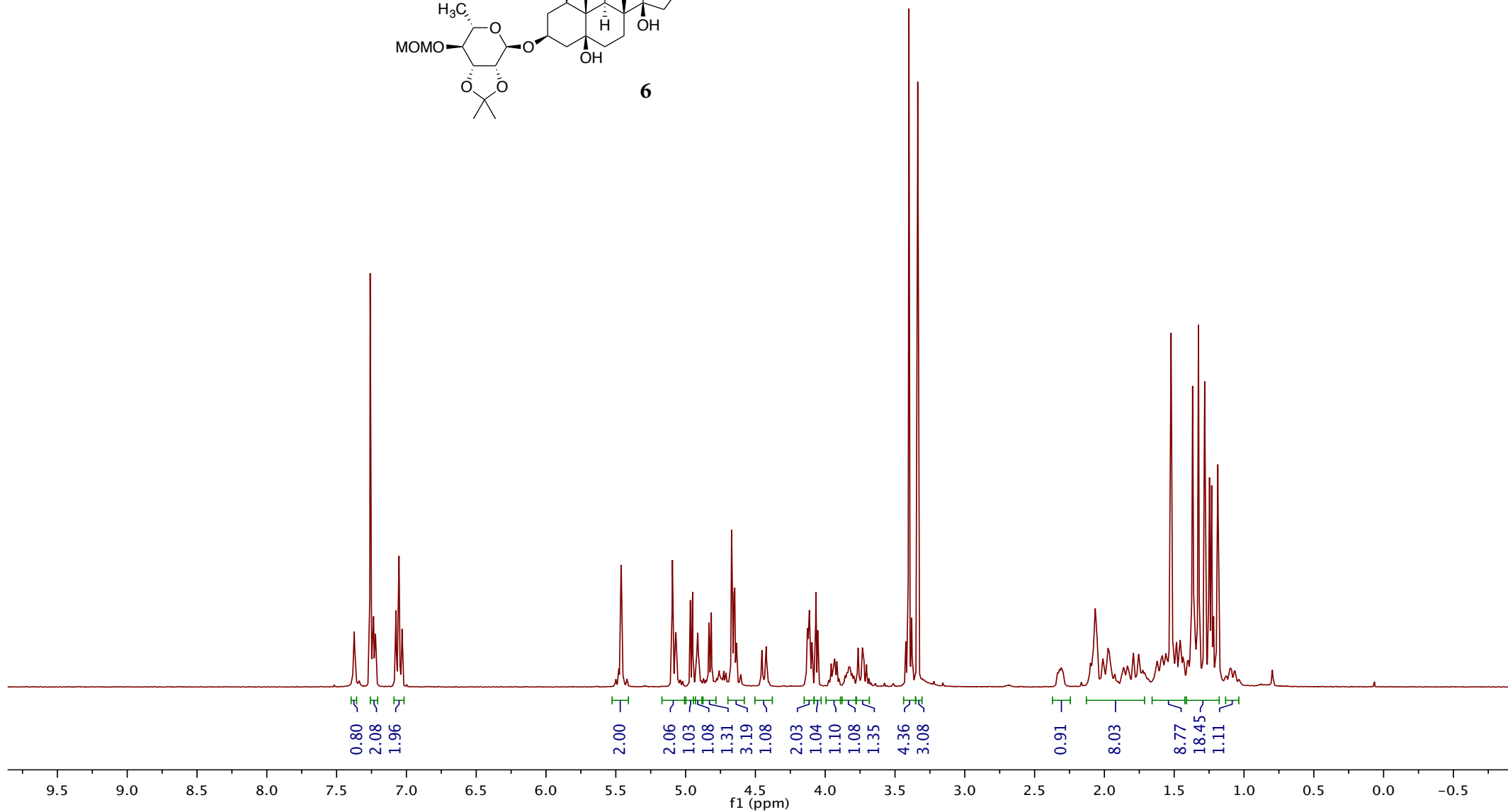
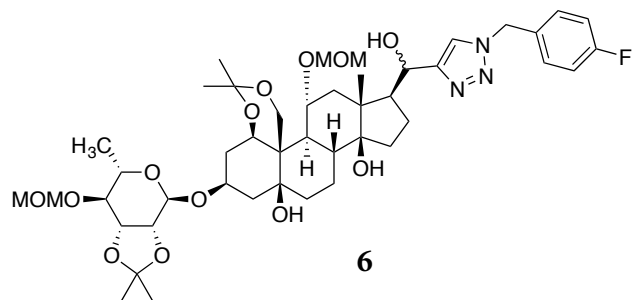
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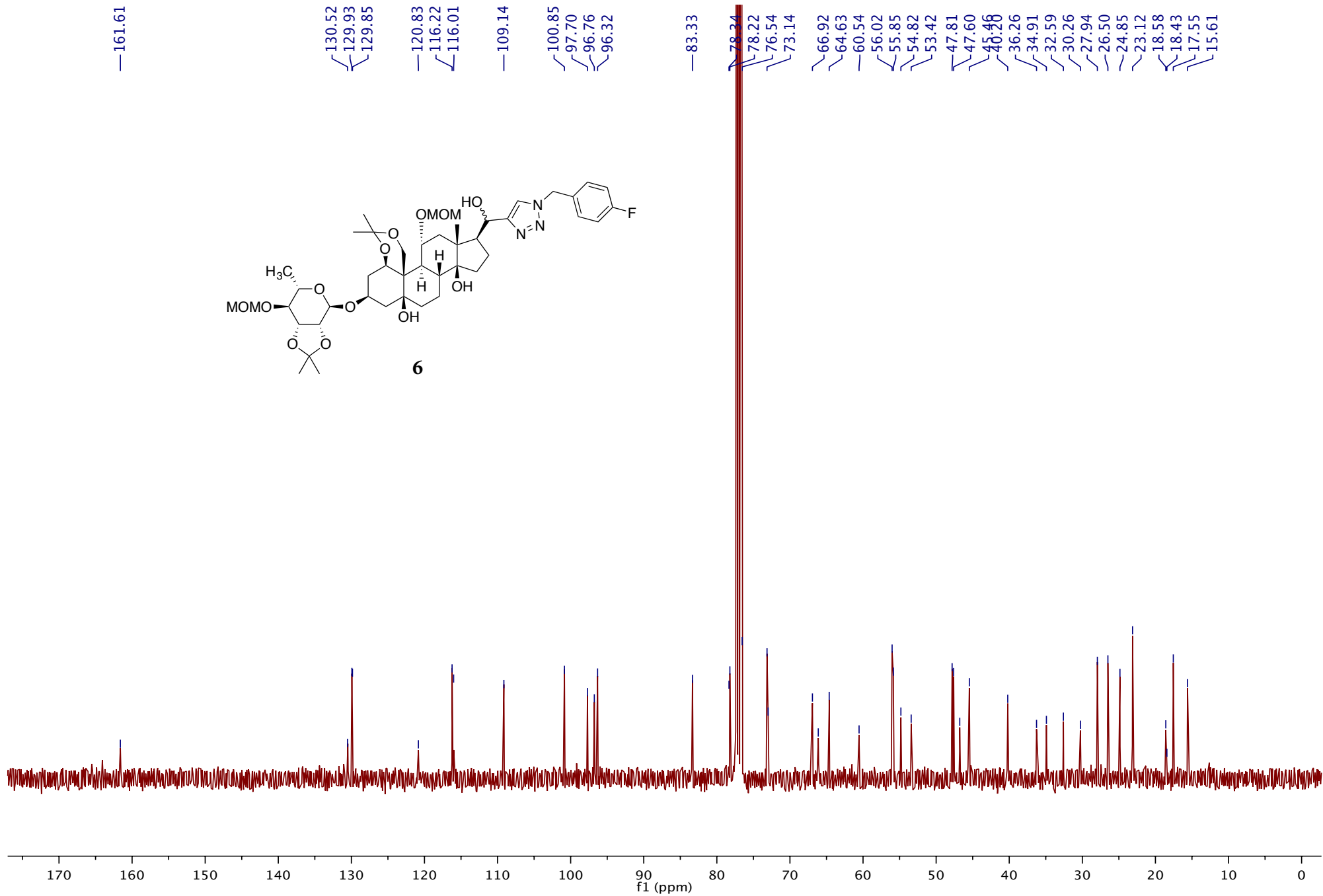


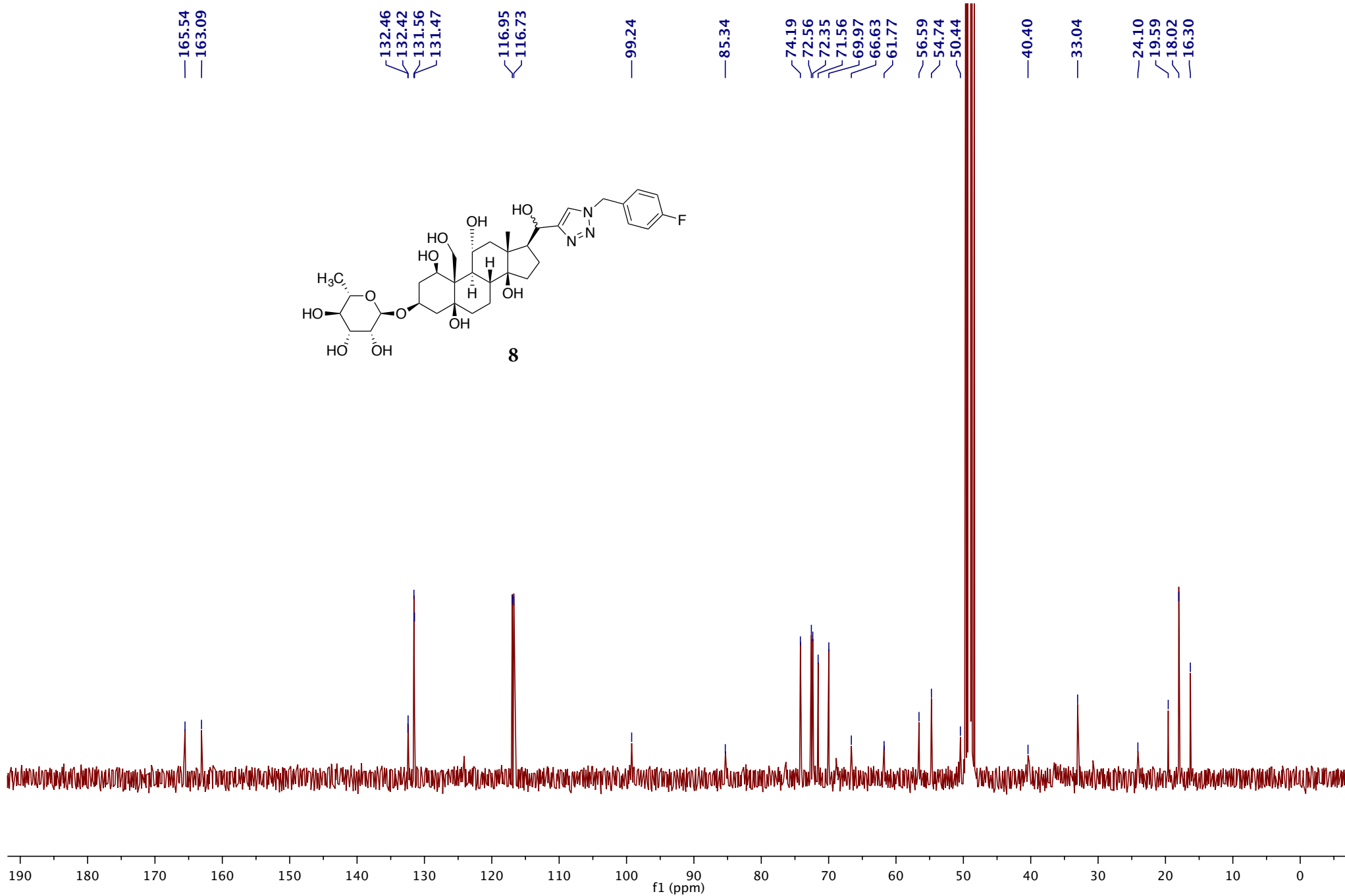
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— 96.32
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— 73.12
— 72.93
— 66.85
— 64.65
— 61.10
— 60.59
— 56.00
— 55.86
— 49.95
— 47.82
— 45.60
— 44.90
— 40.09
— 36.21
— 34.88
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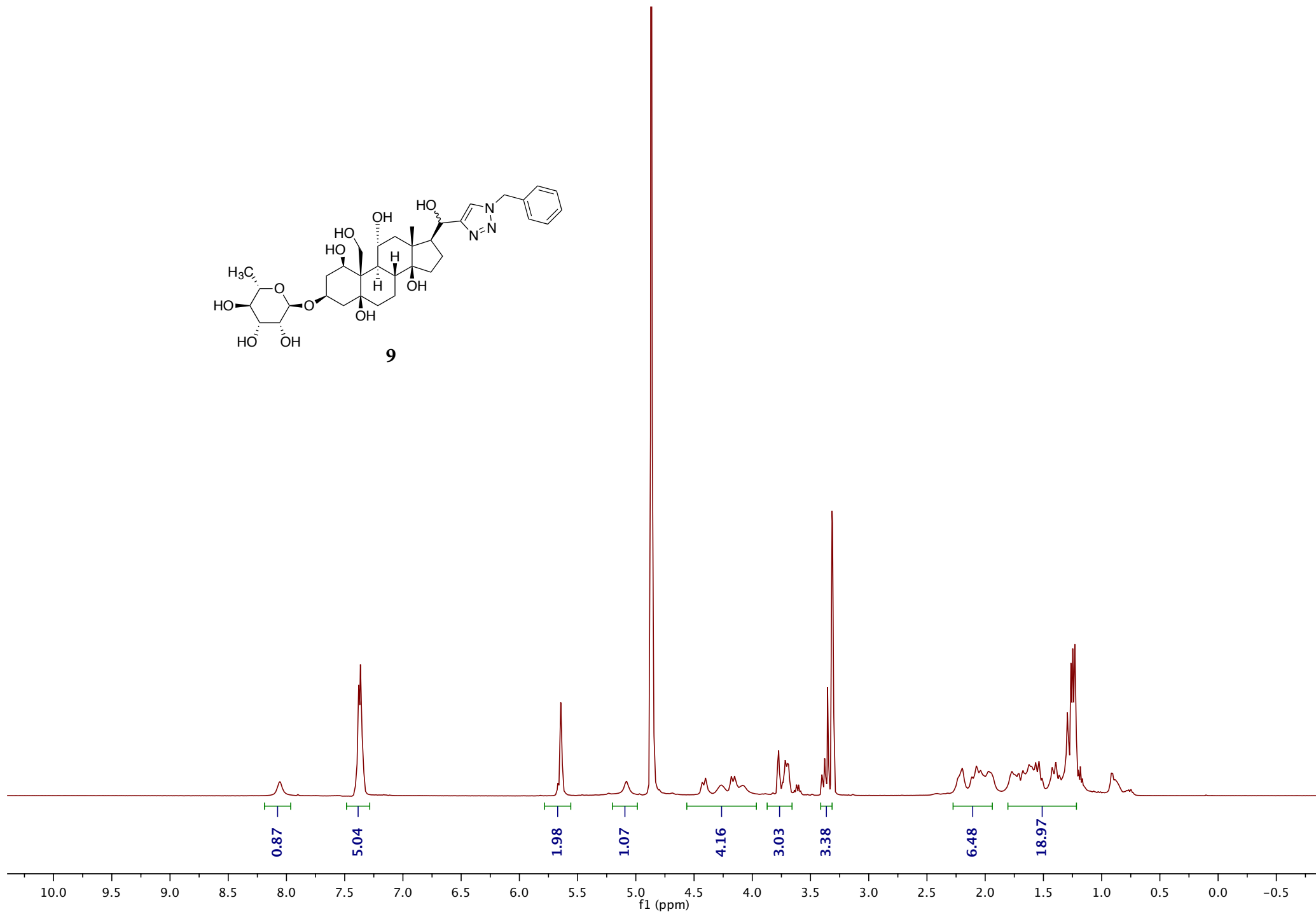
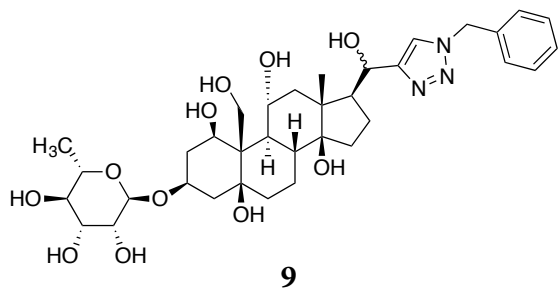


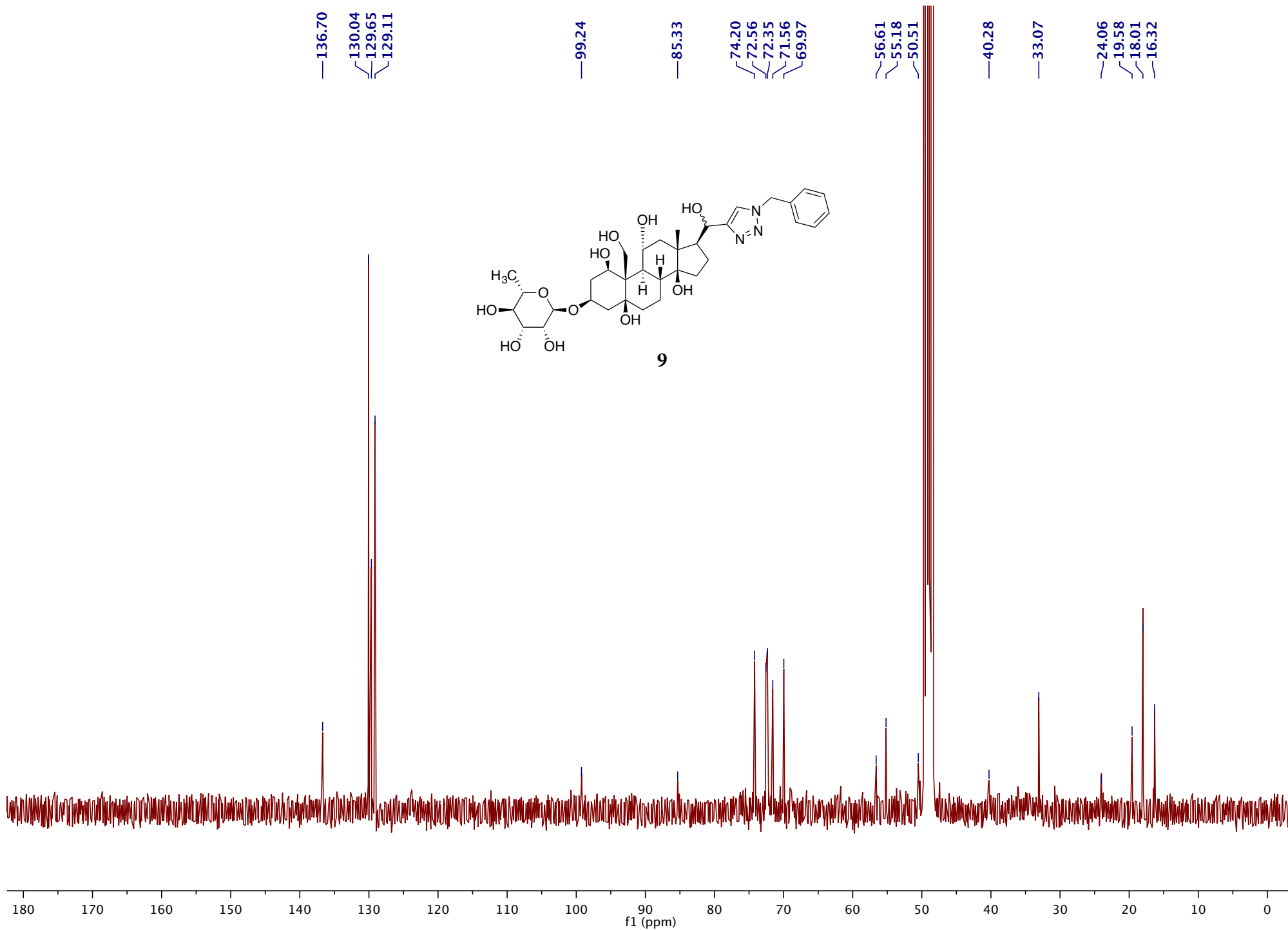


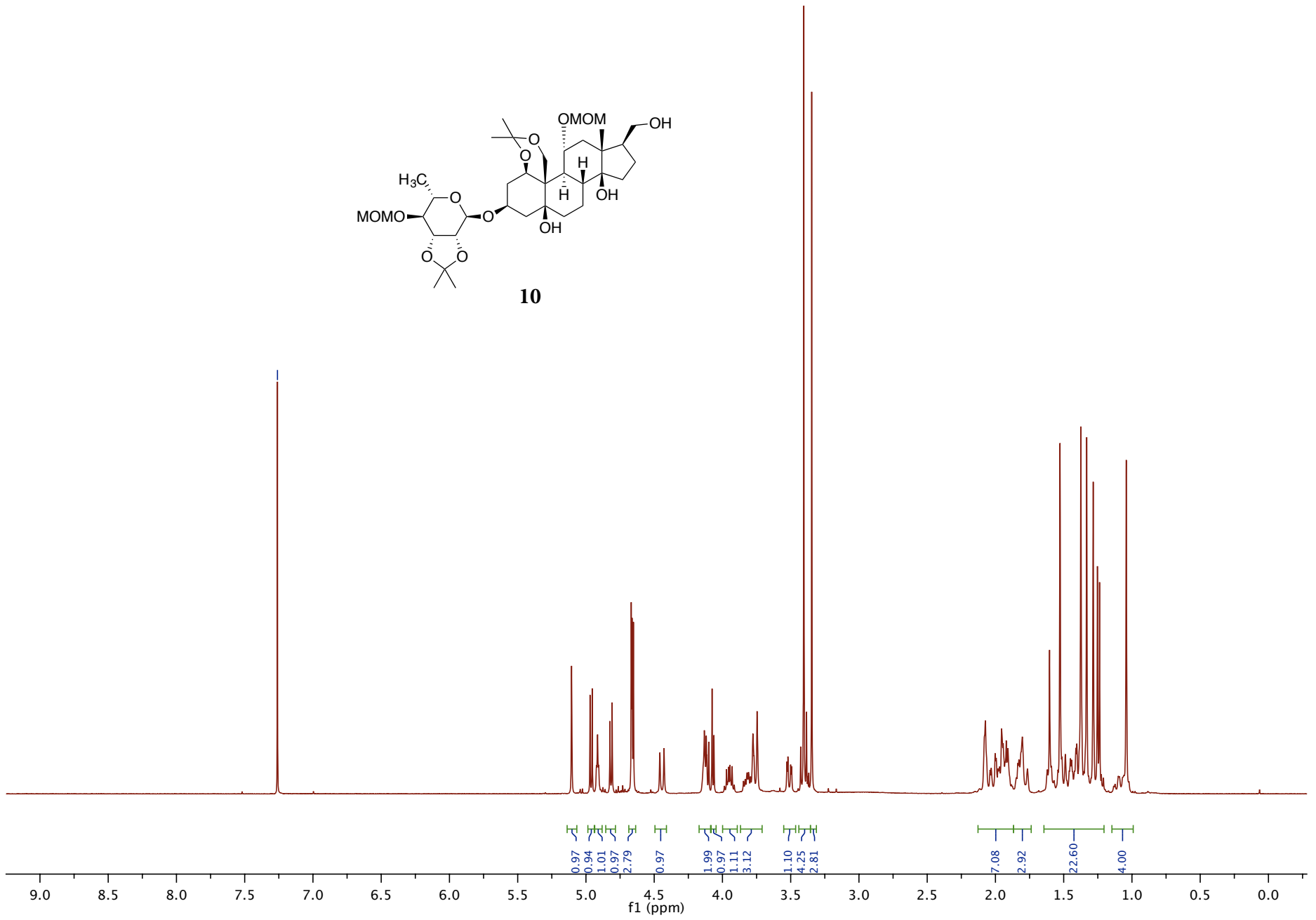
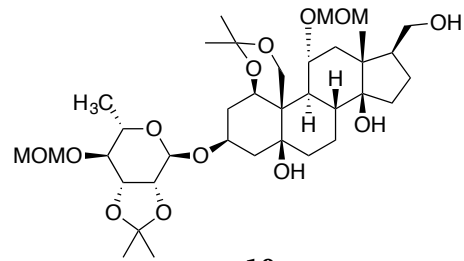


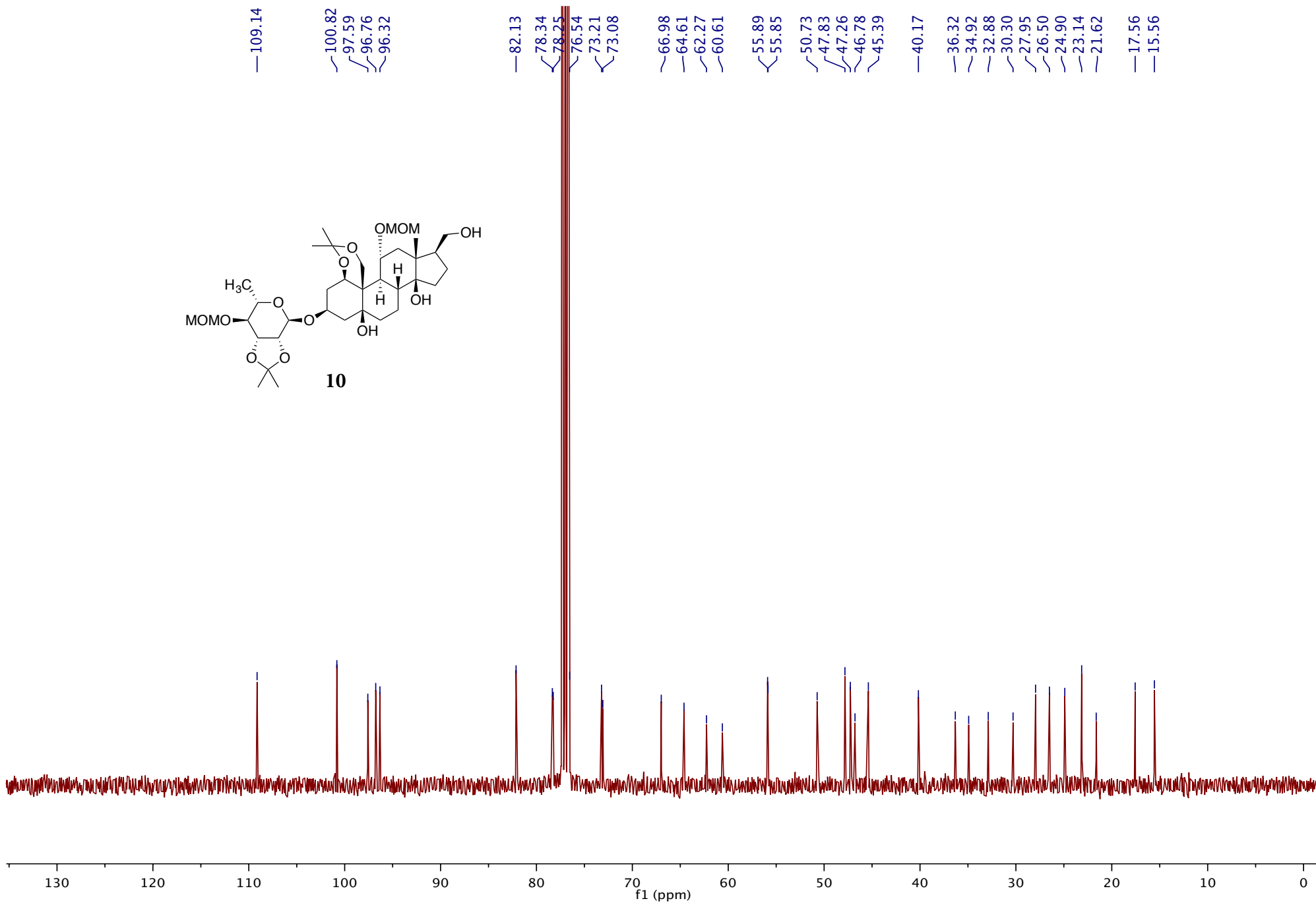
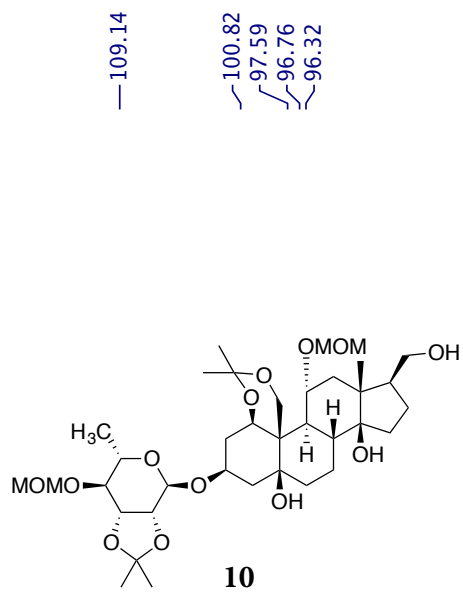


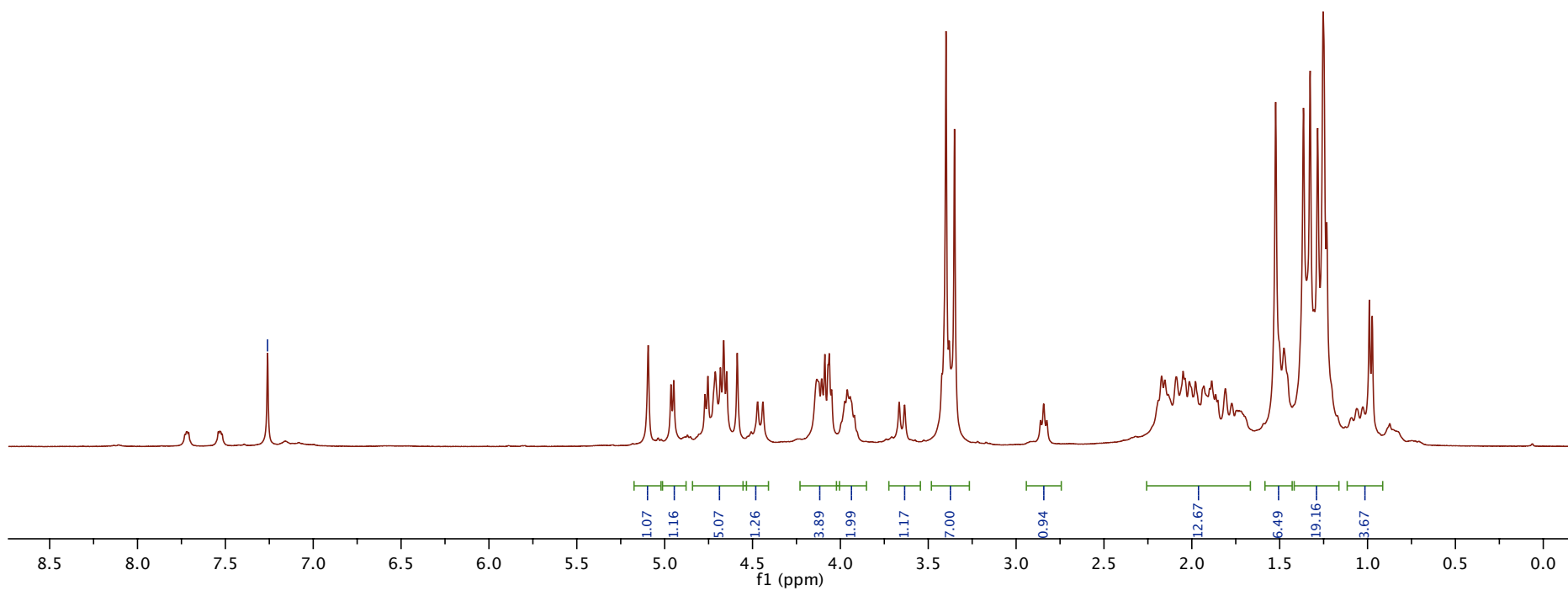
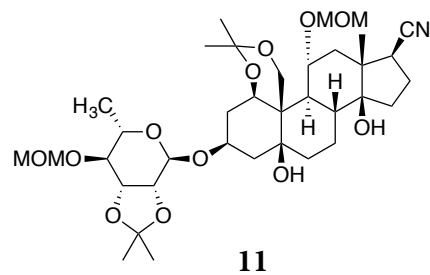


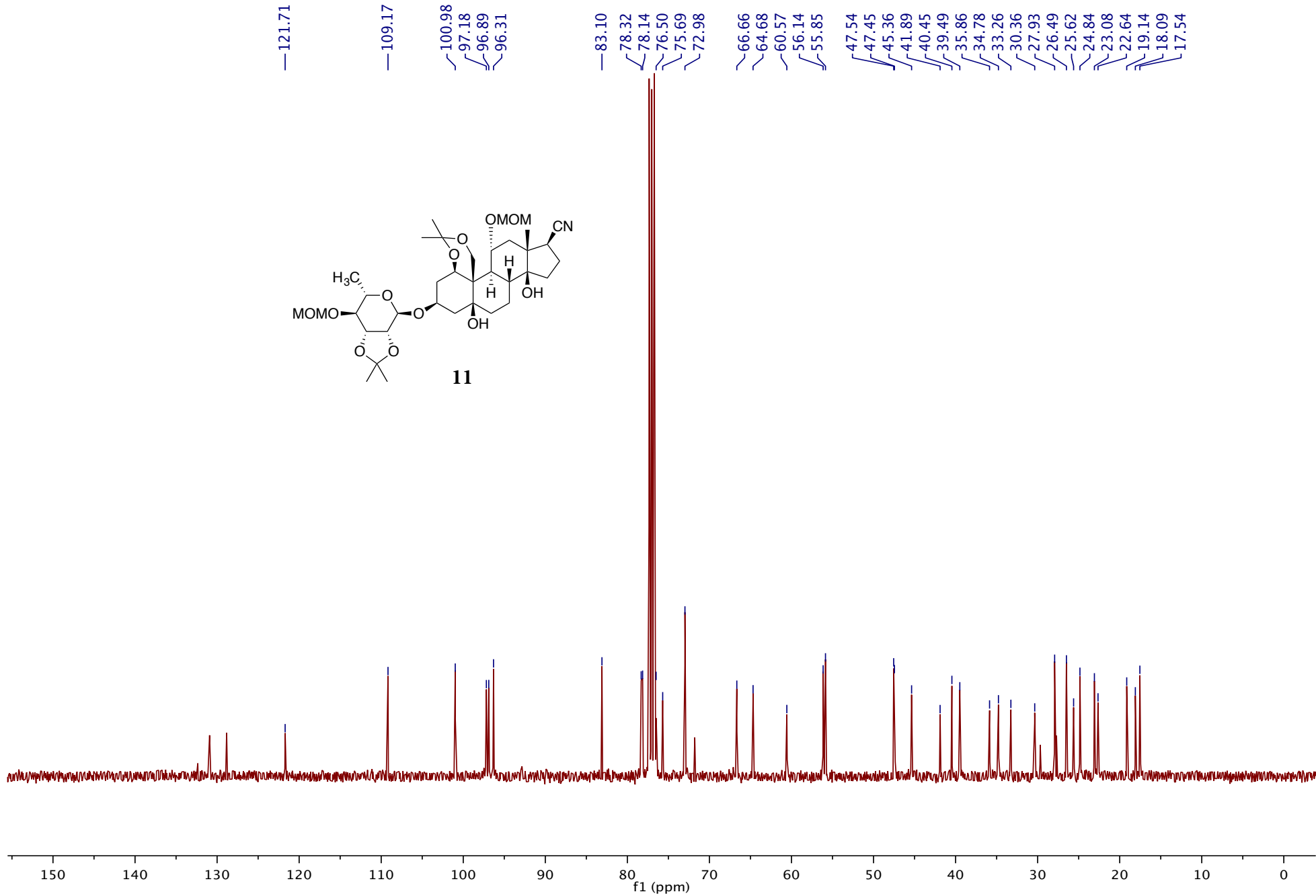




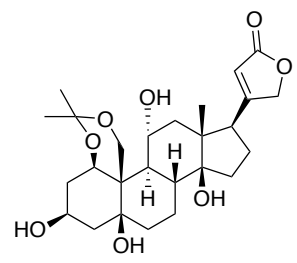




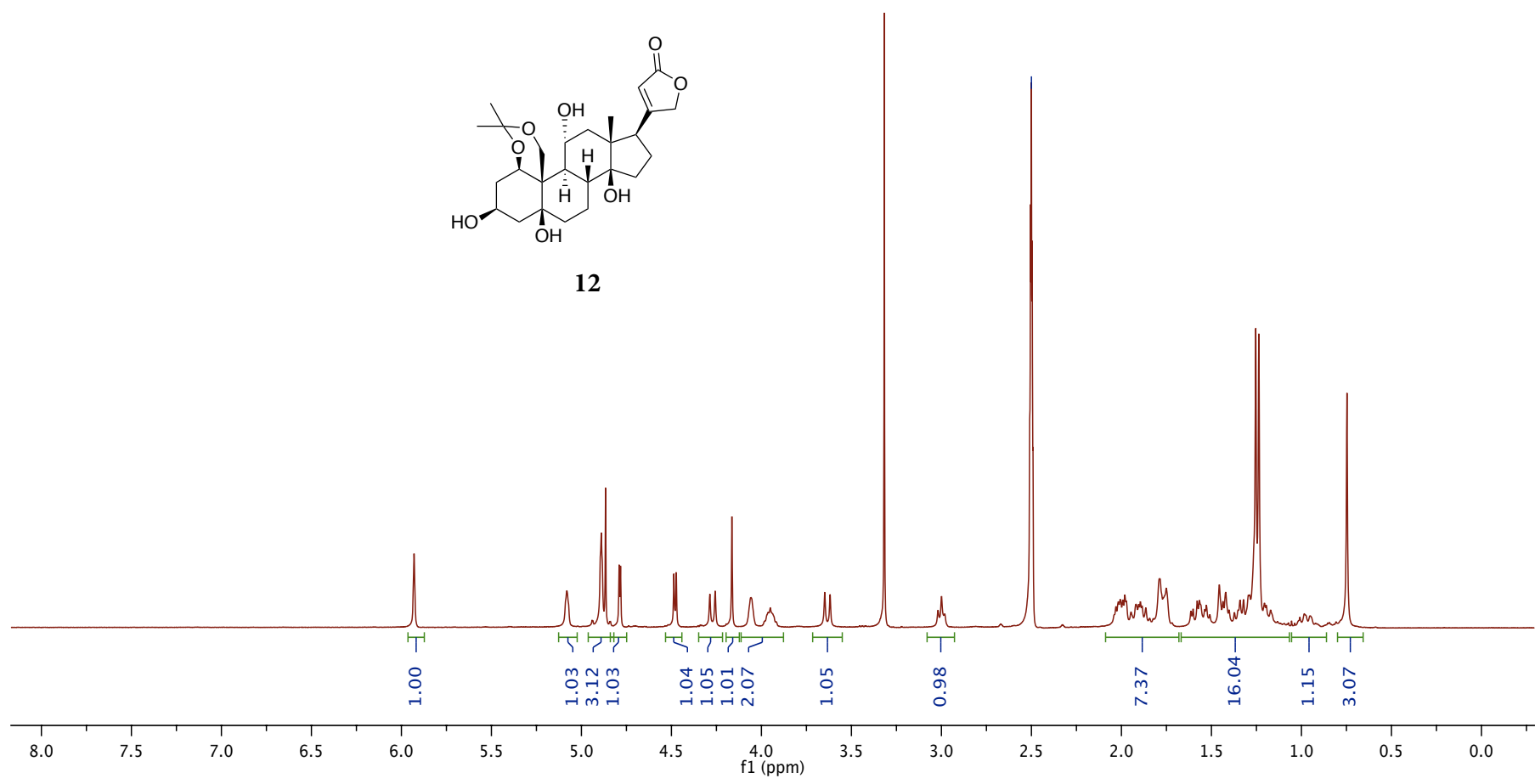


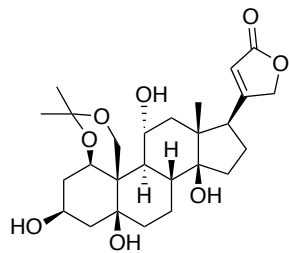


— 2.50 DMSO

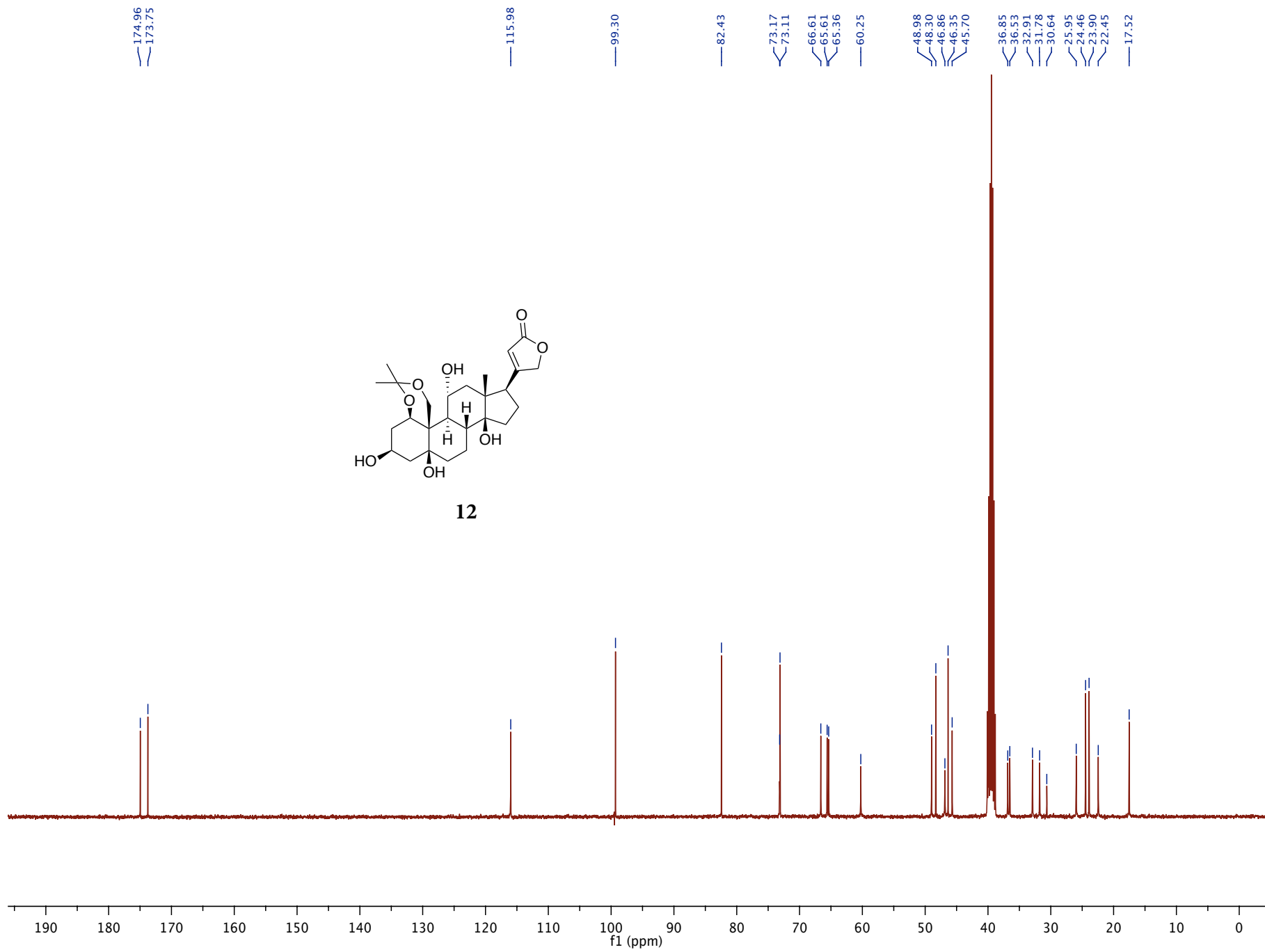


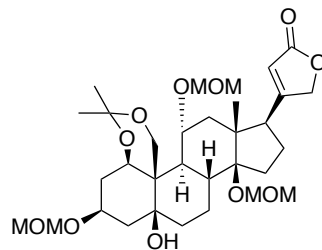
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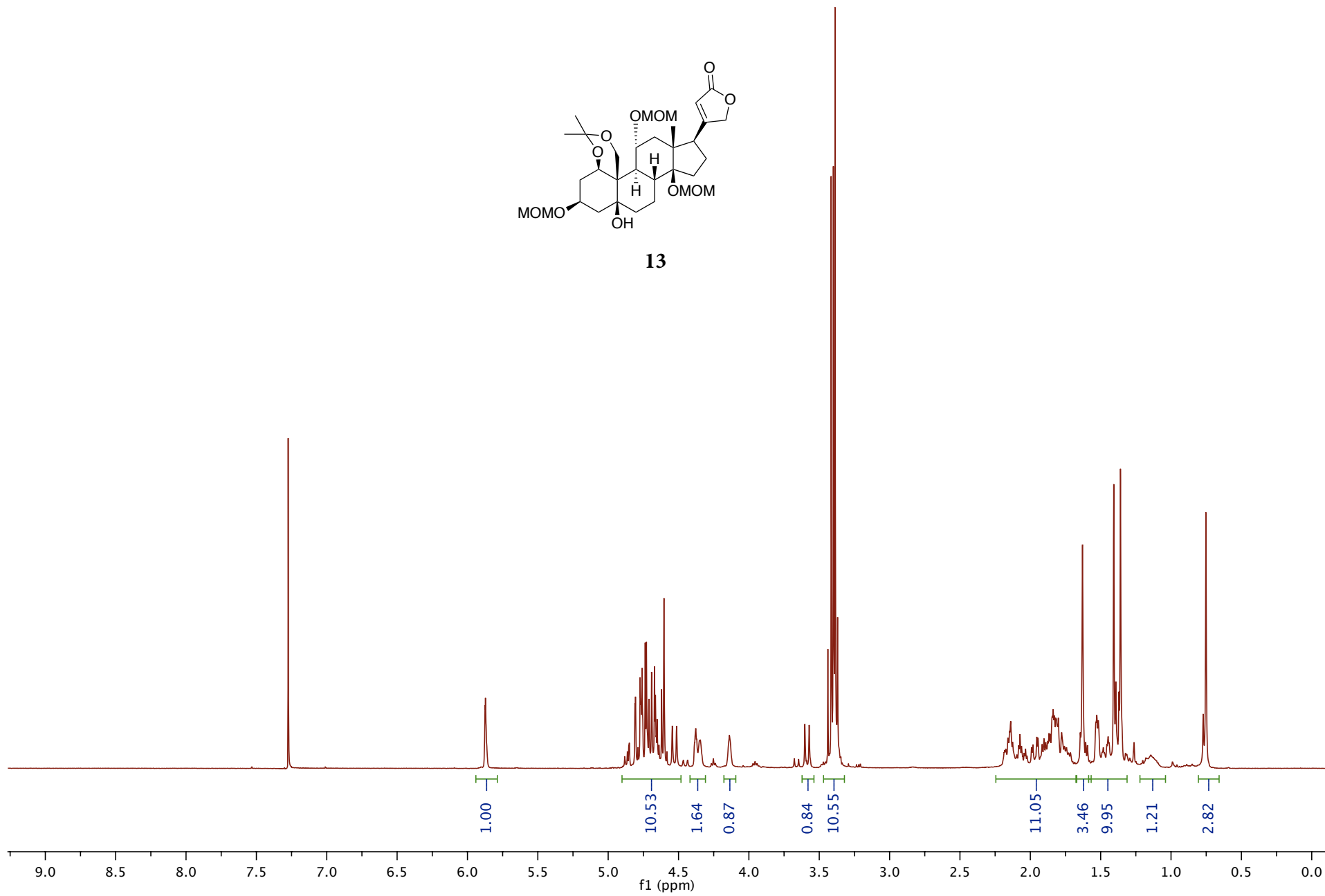


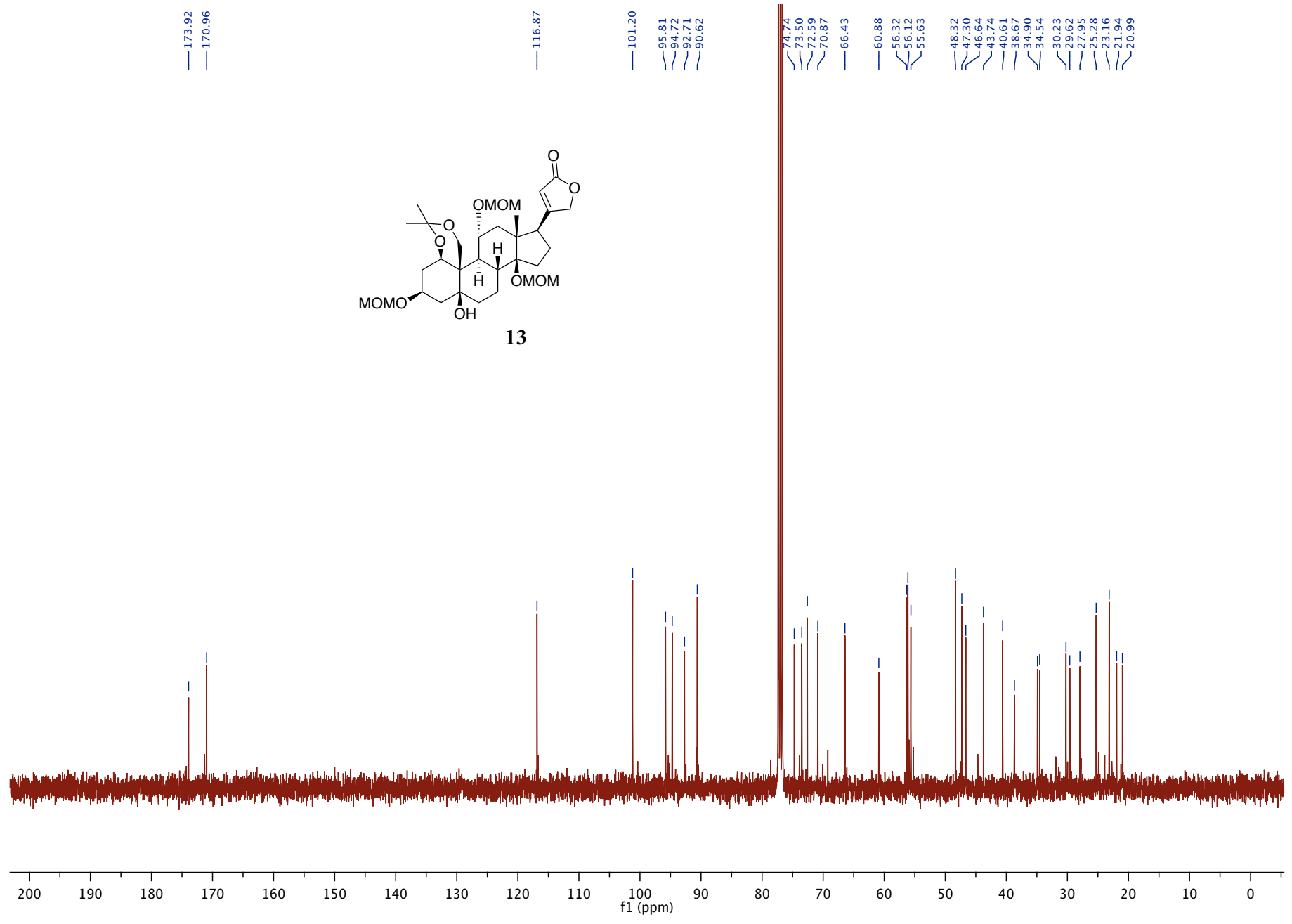
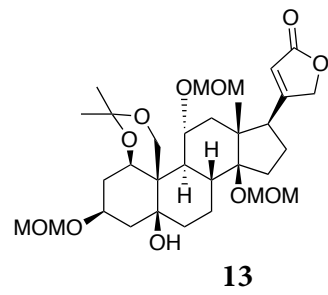
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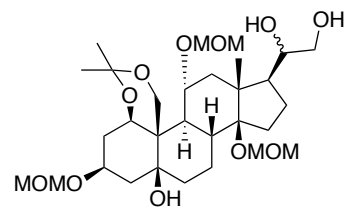




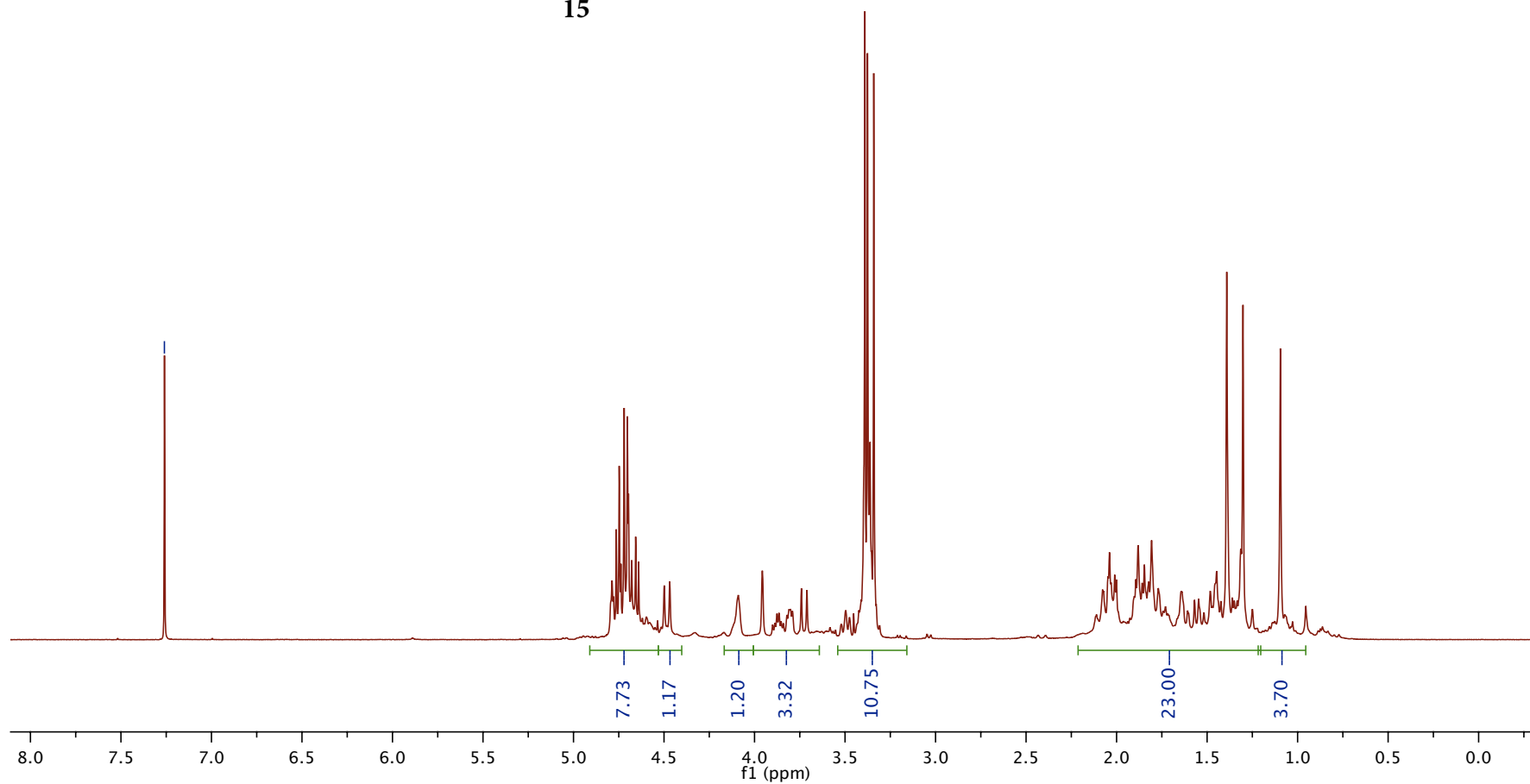
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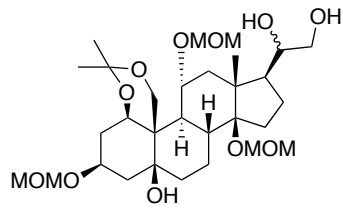




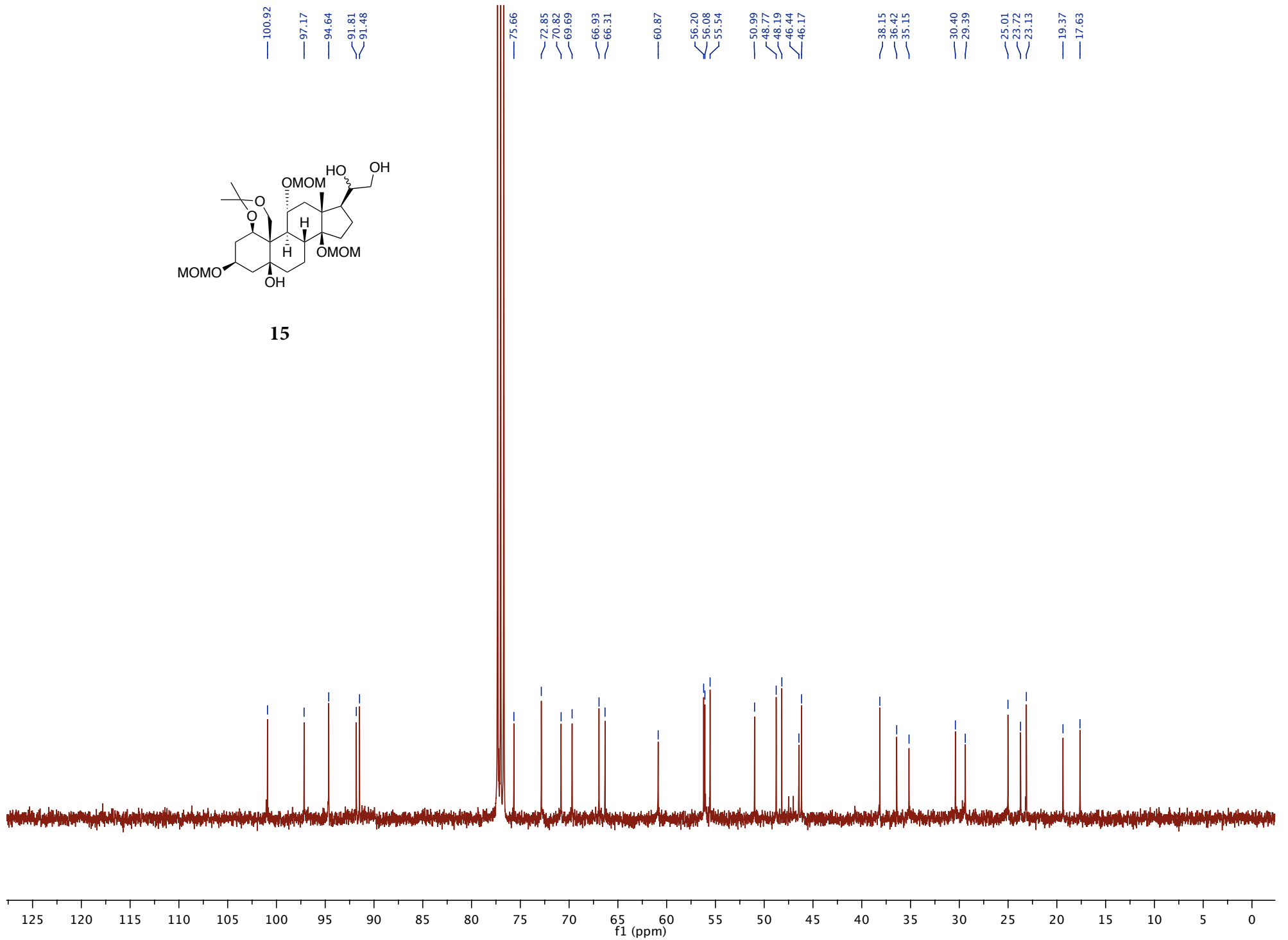


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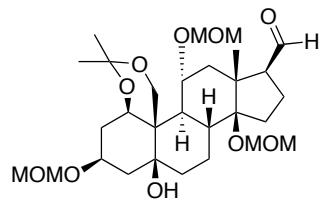




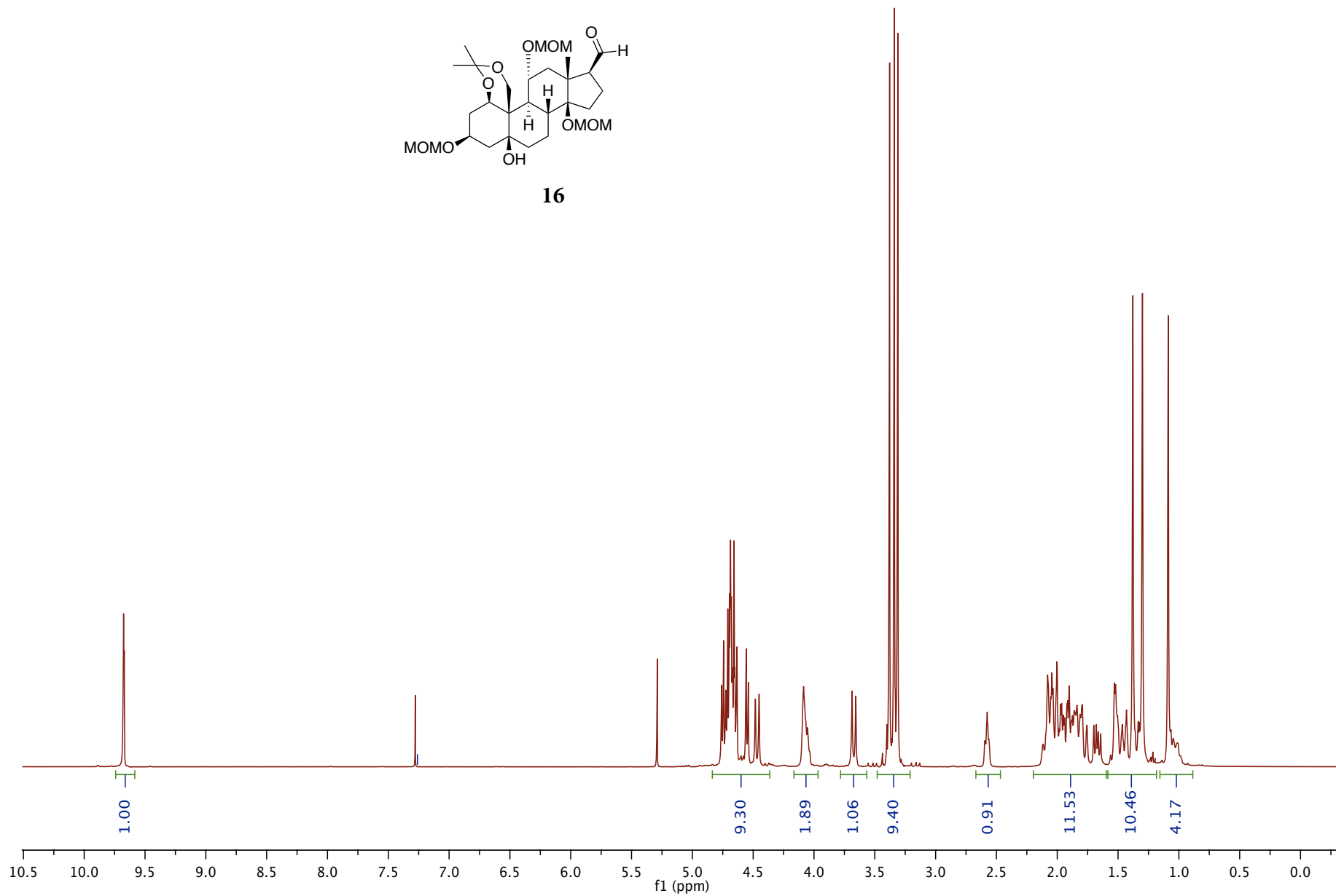
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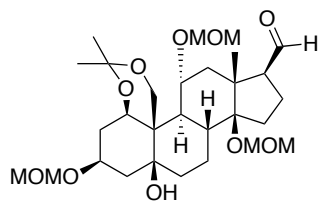
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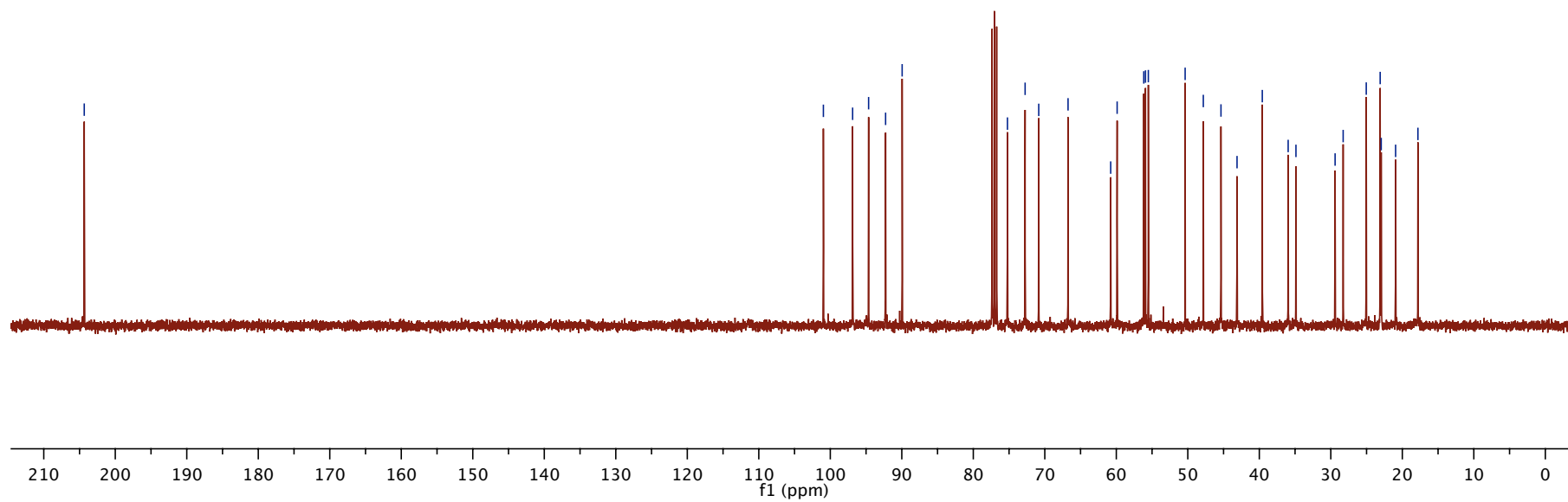
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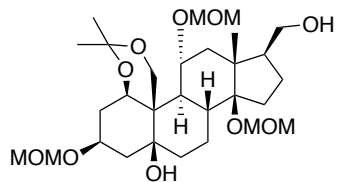


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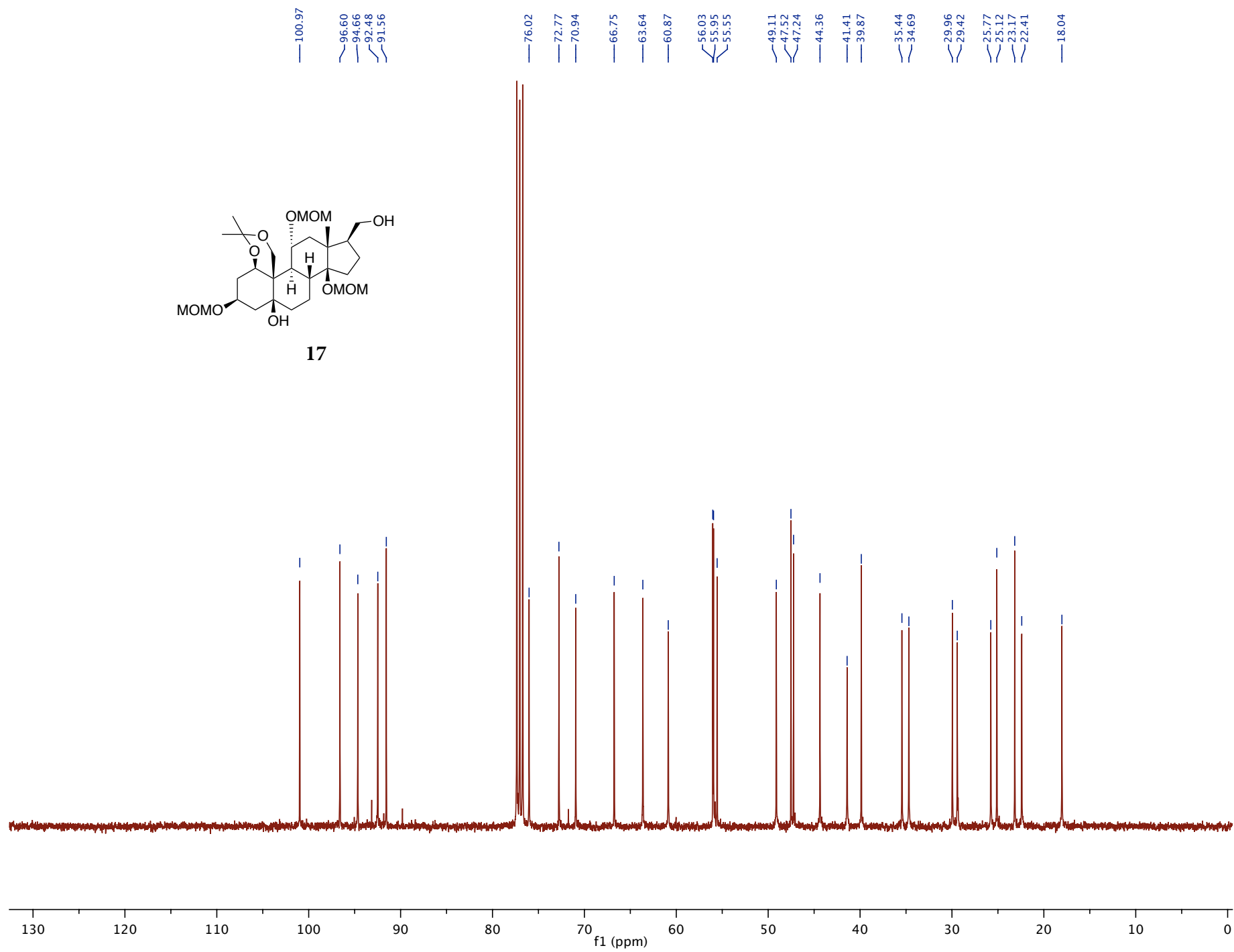


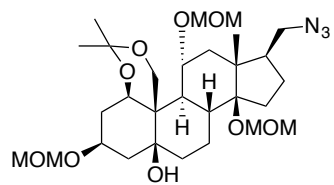
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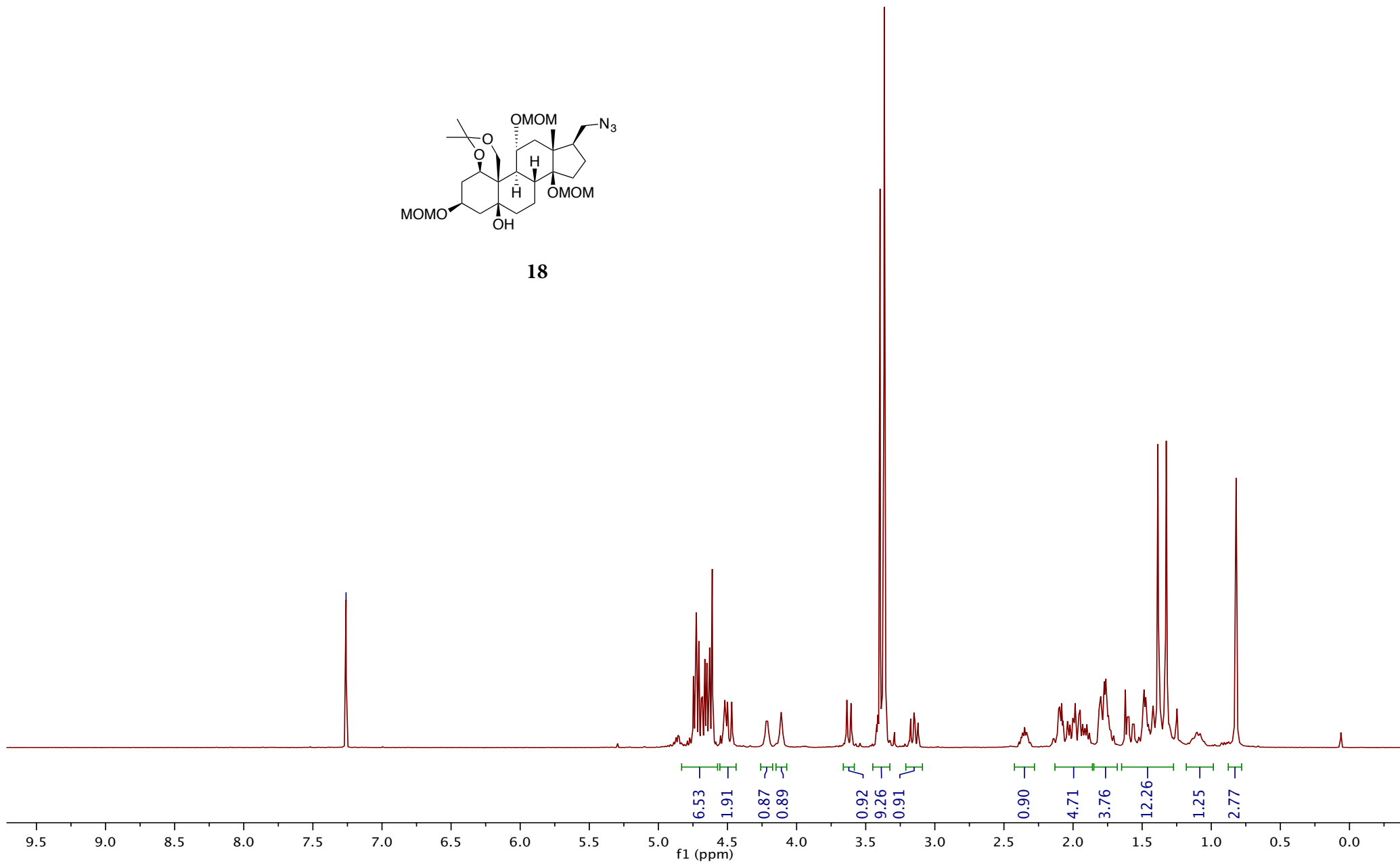


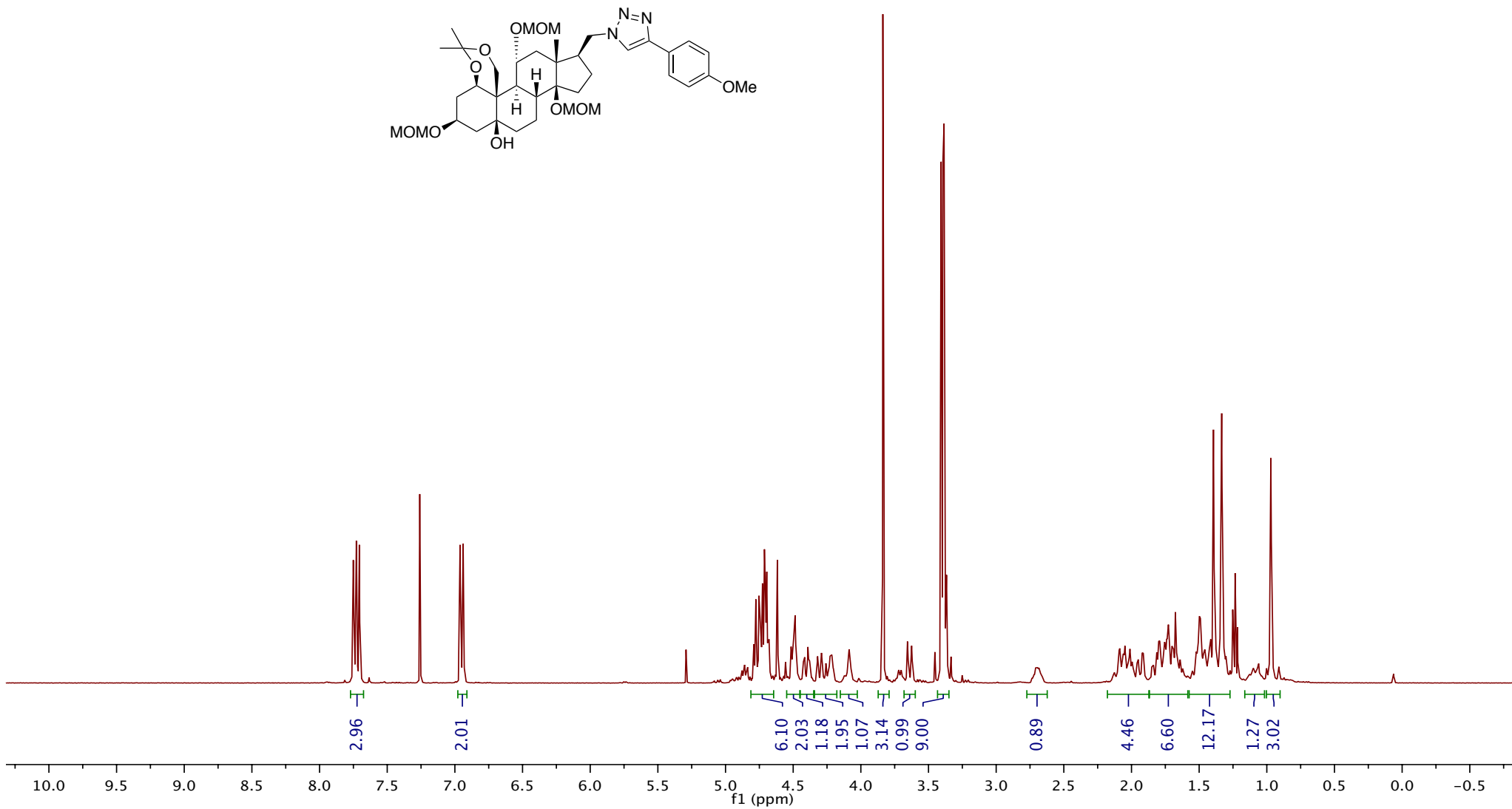
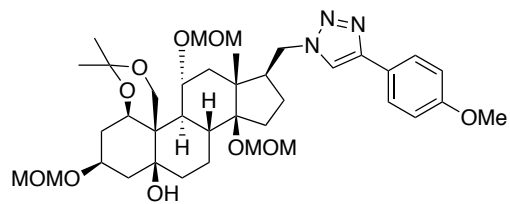
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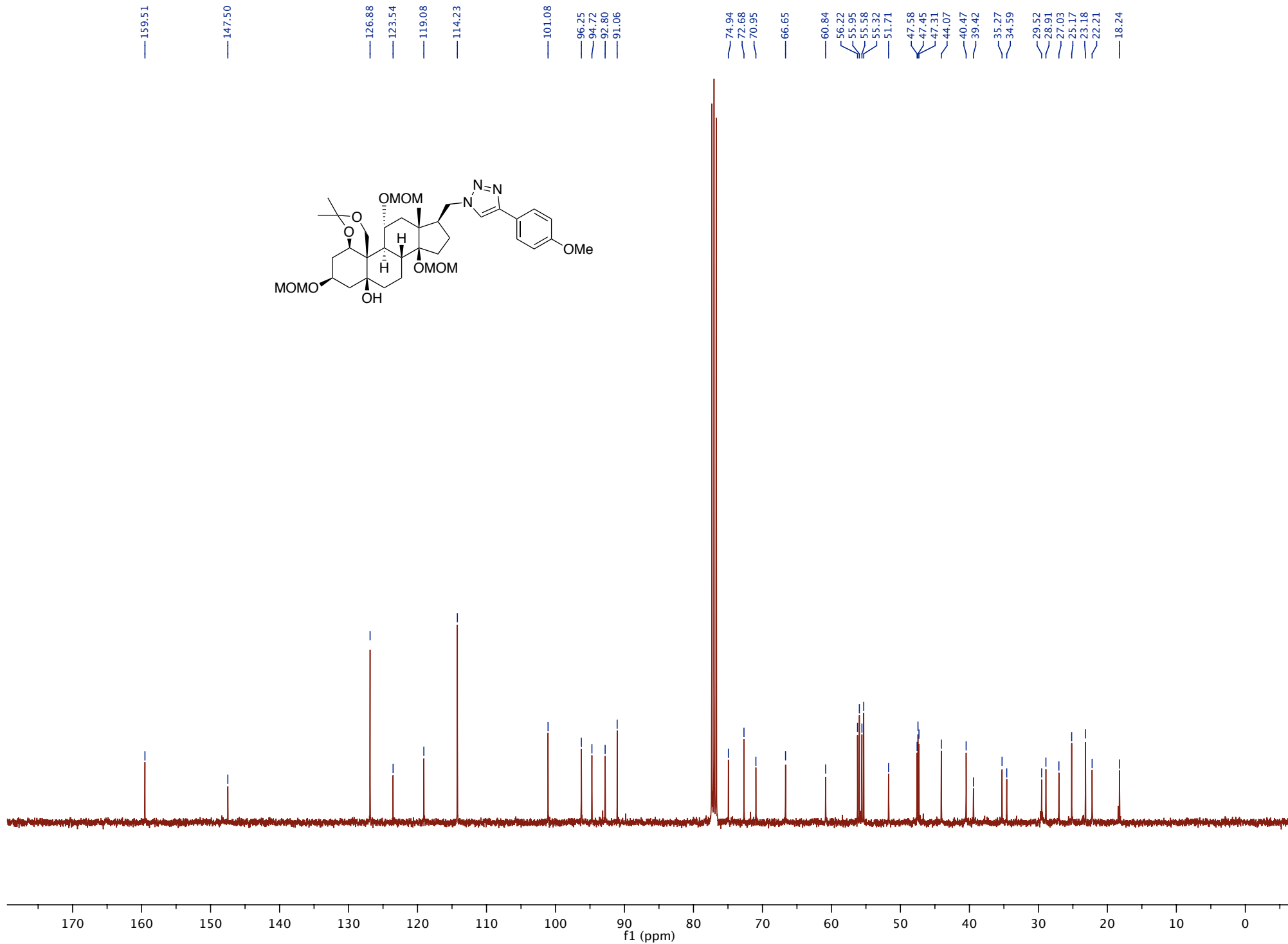
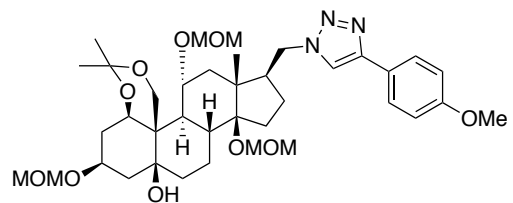


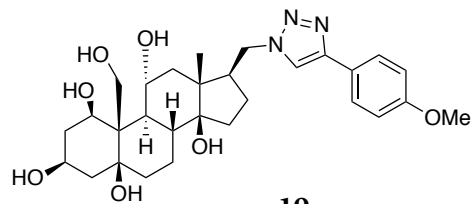


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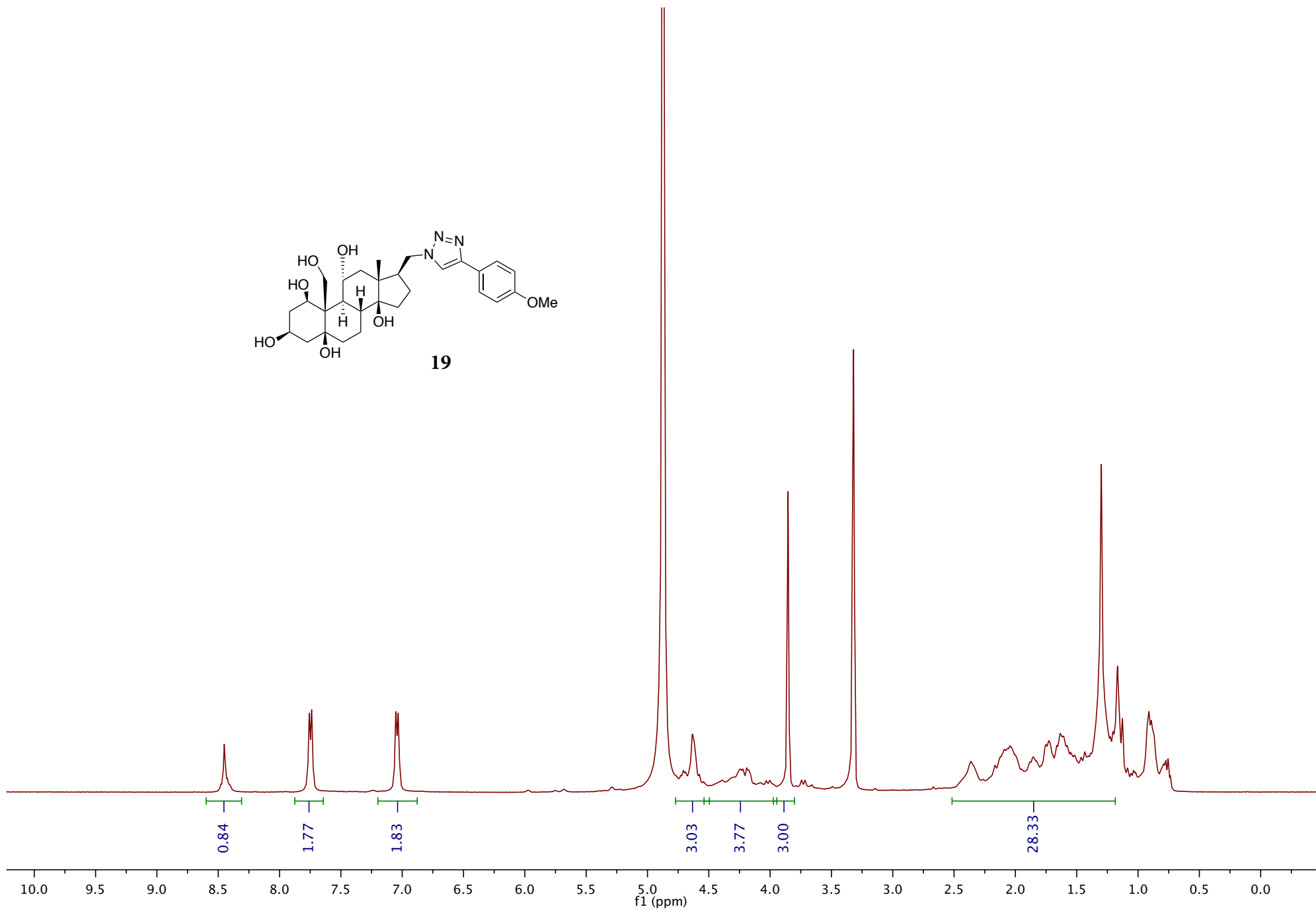


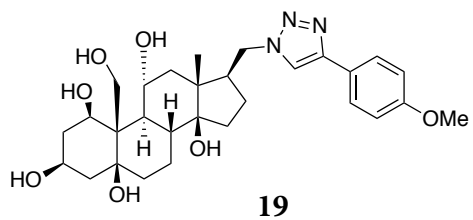






19





—162.02

—128.43

—122.69

—122.17

—115.63

—87.18

—78.56

—75.52

—68.60

—62.85

—56.57

—55.89

—51.85

—50.87

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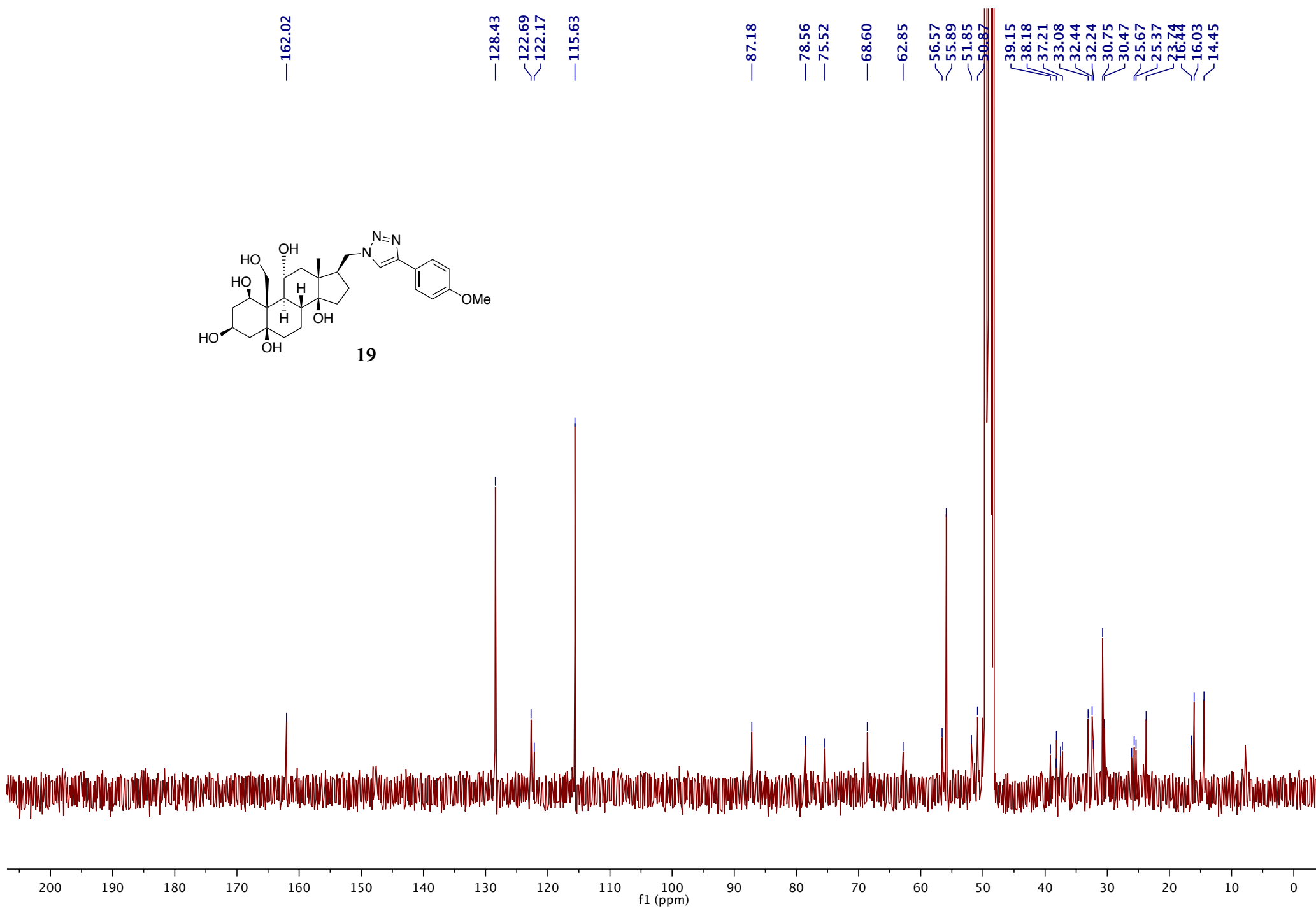
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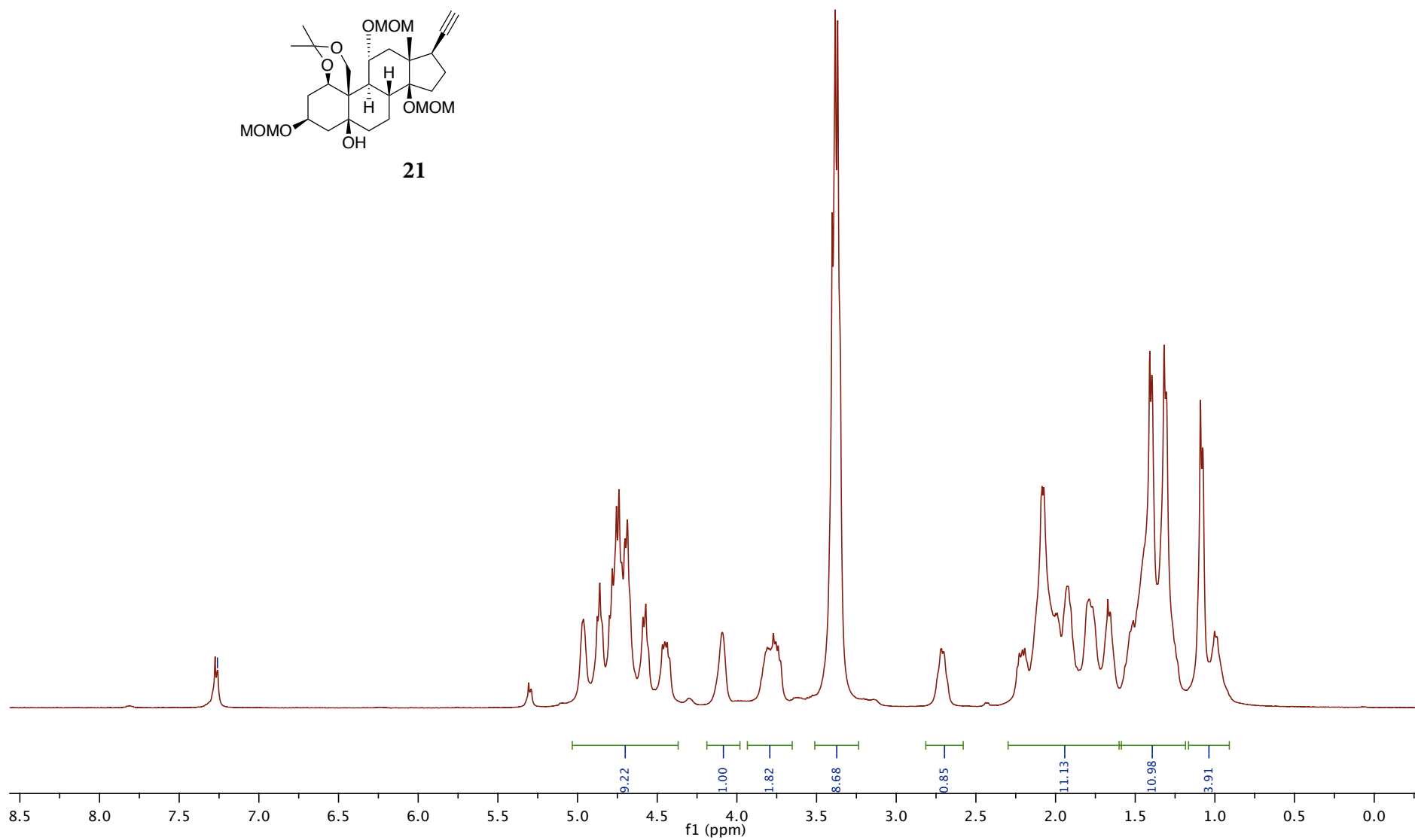
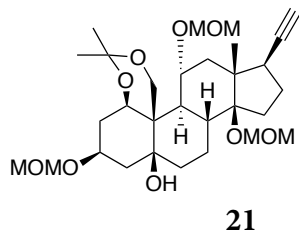
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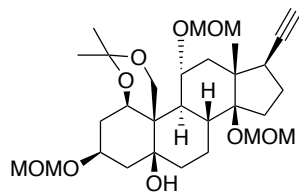
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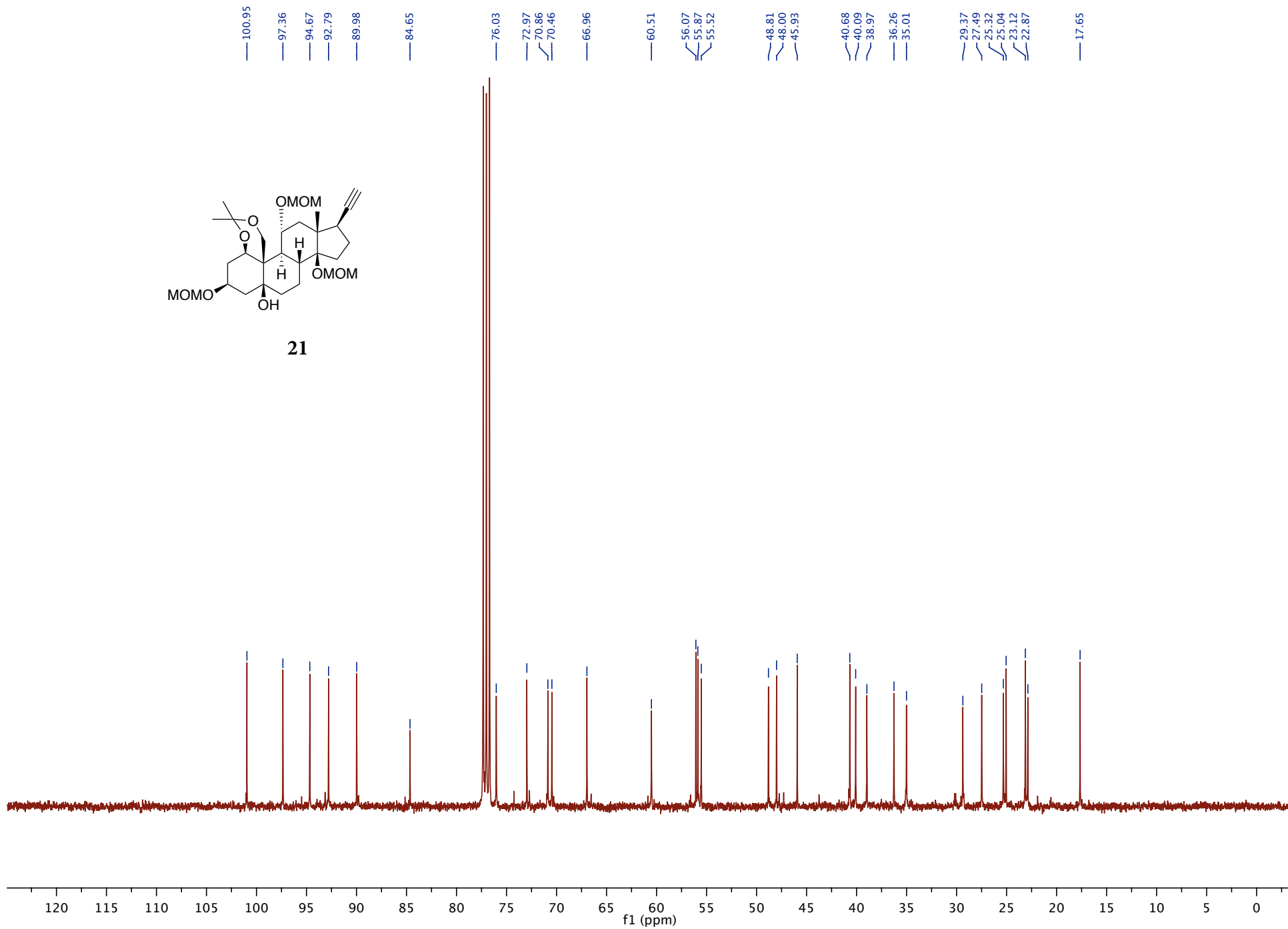
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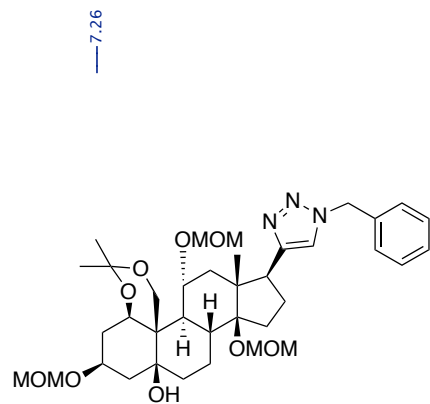
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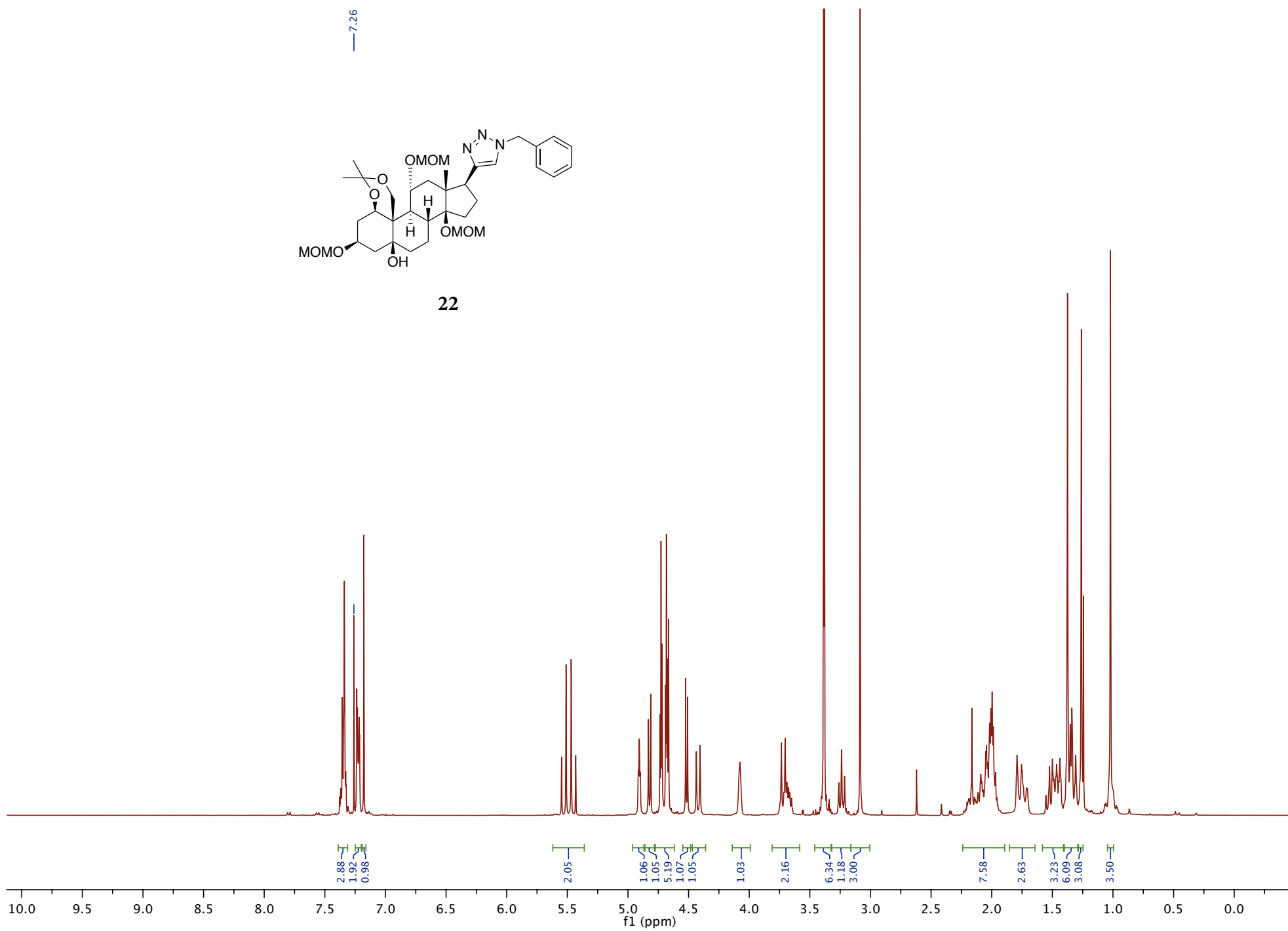


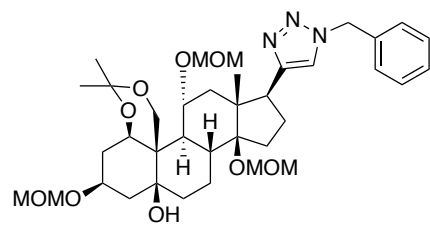
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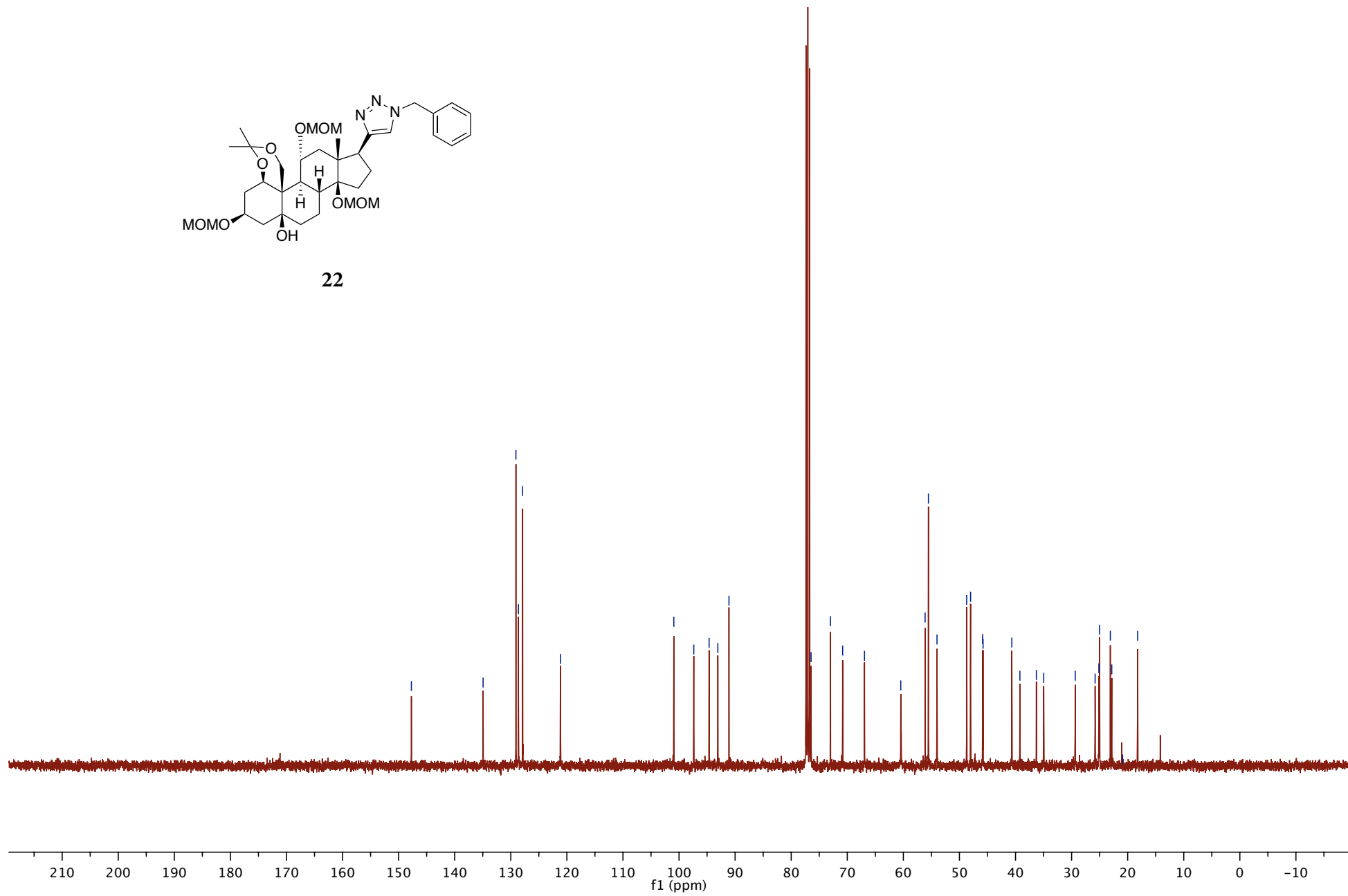
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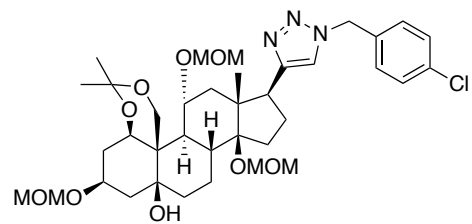




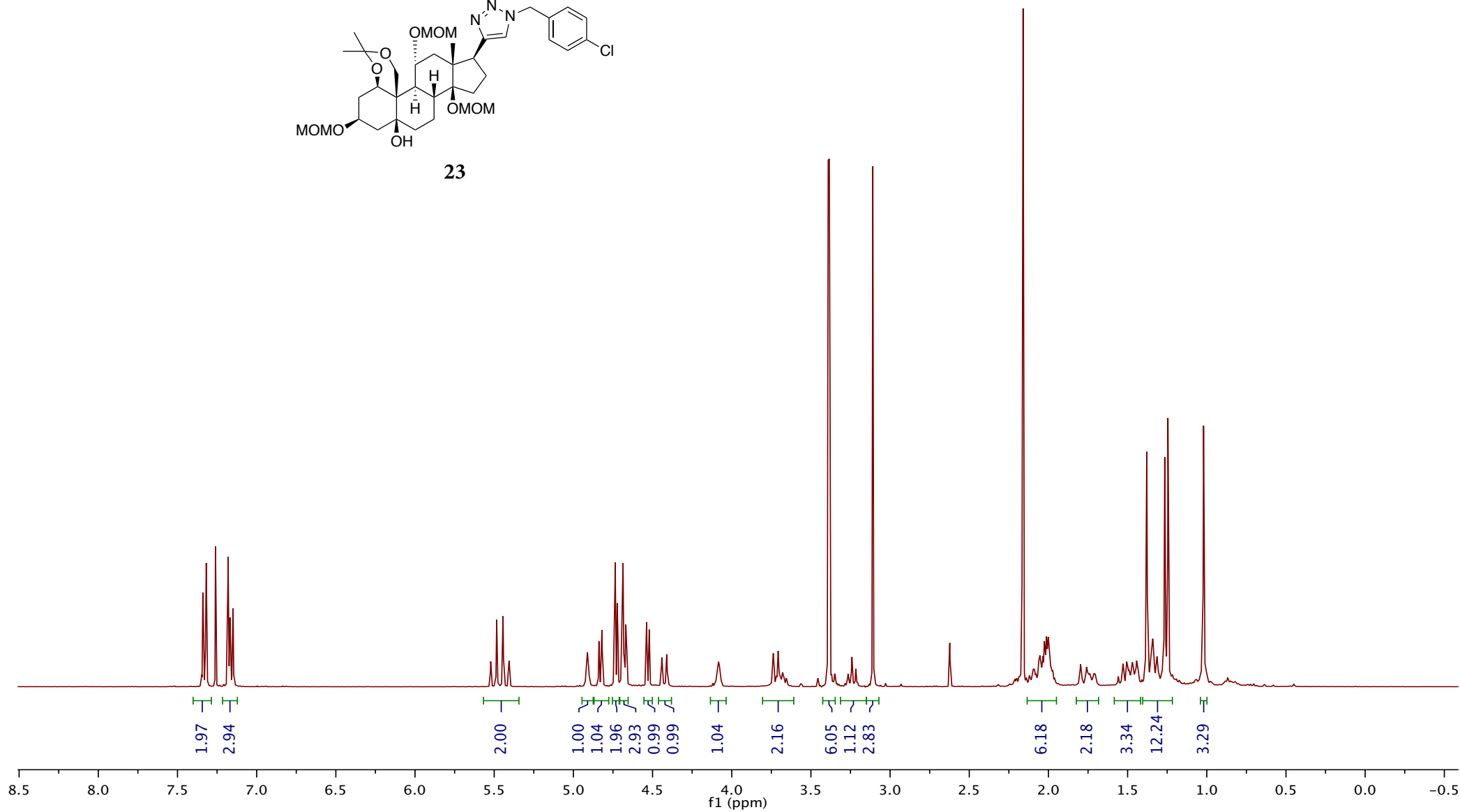
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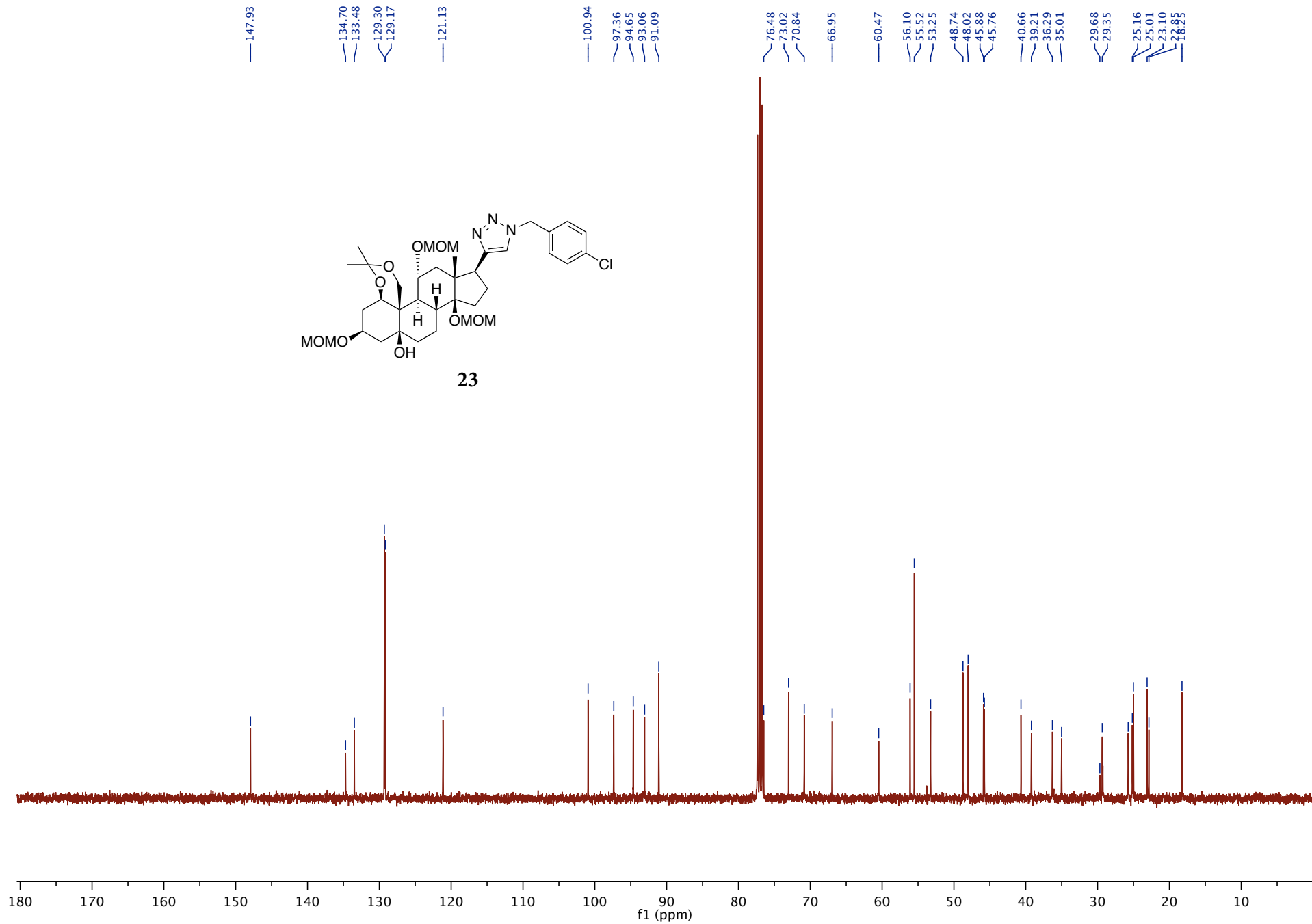
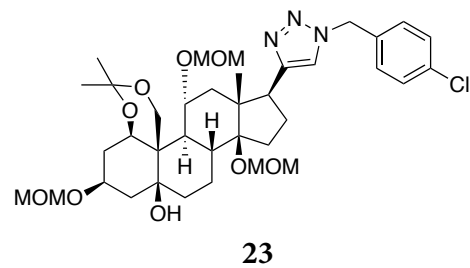


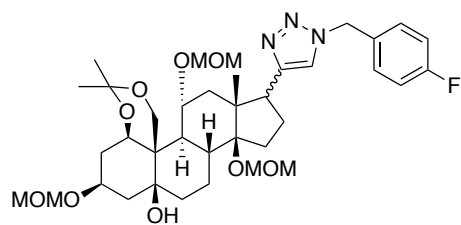


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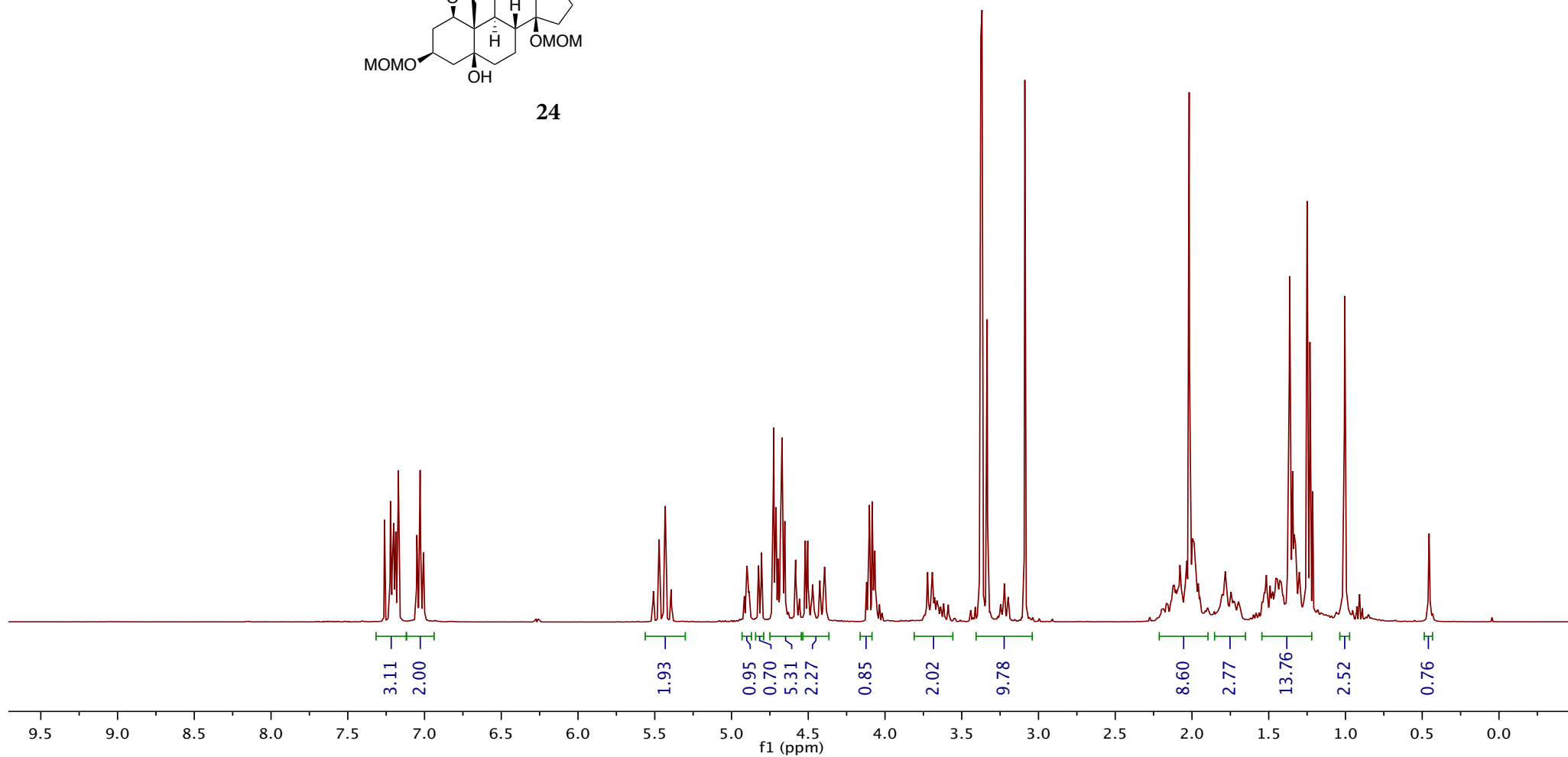


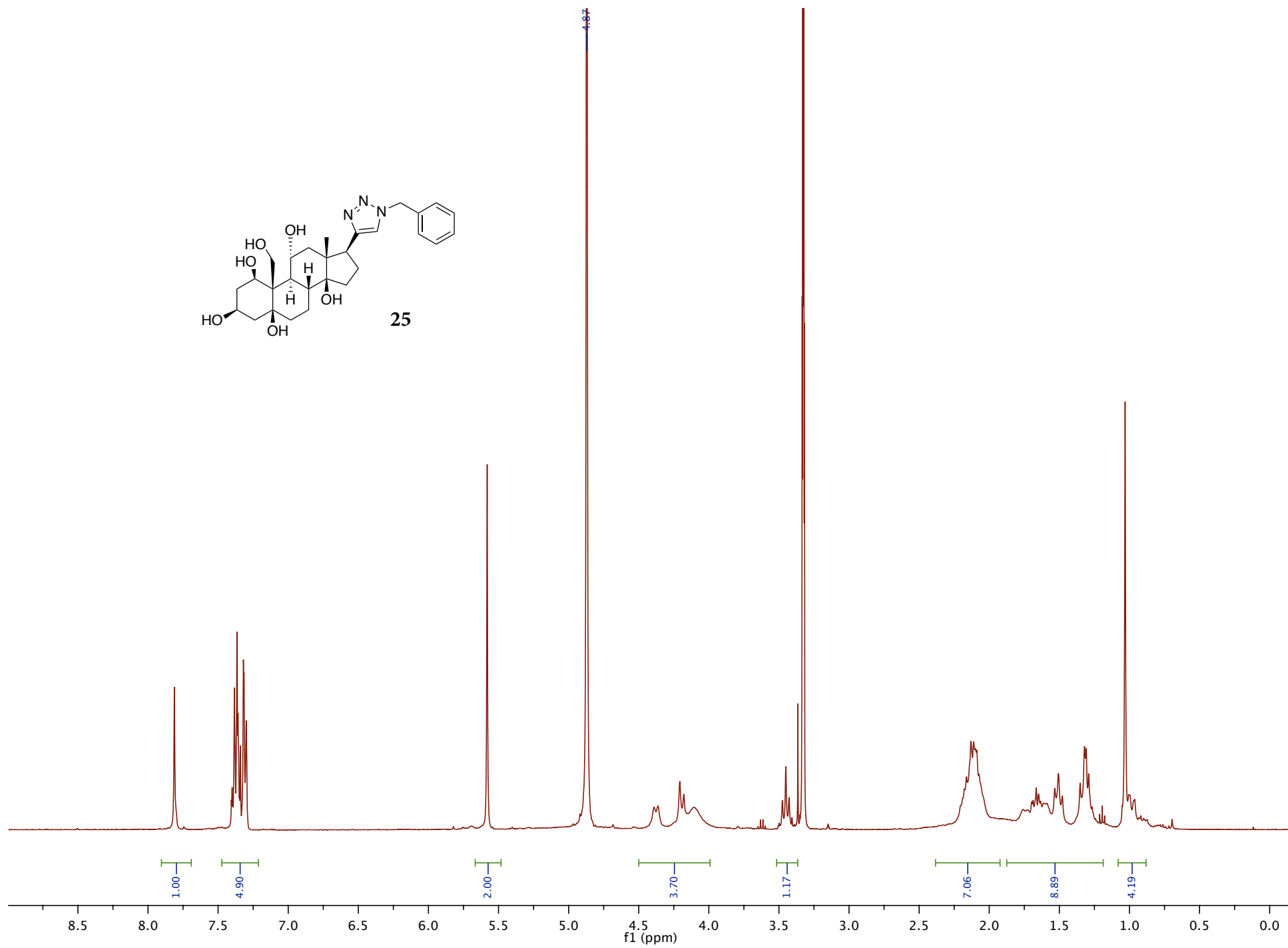
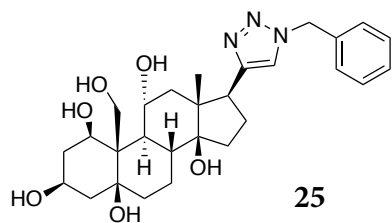
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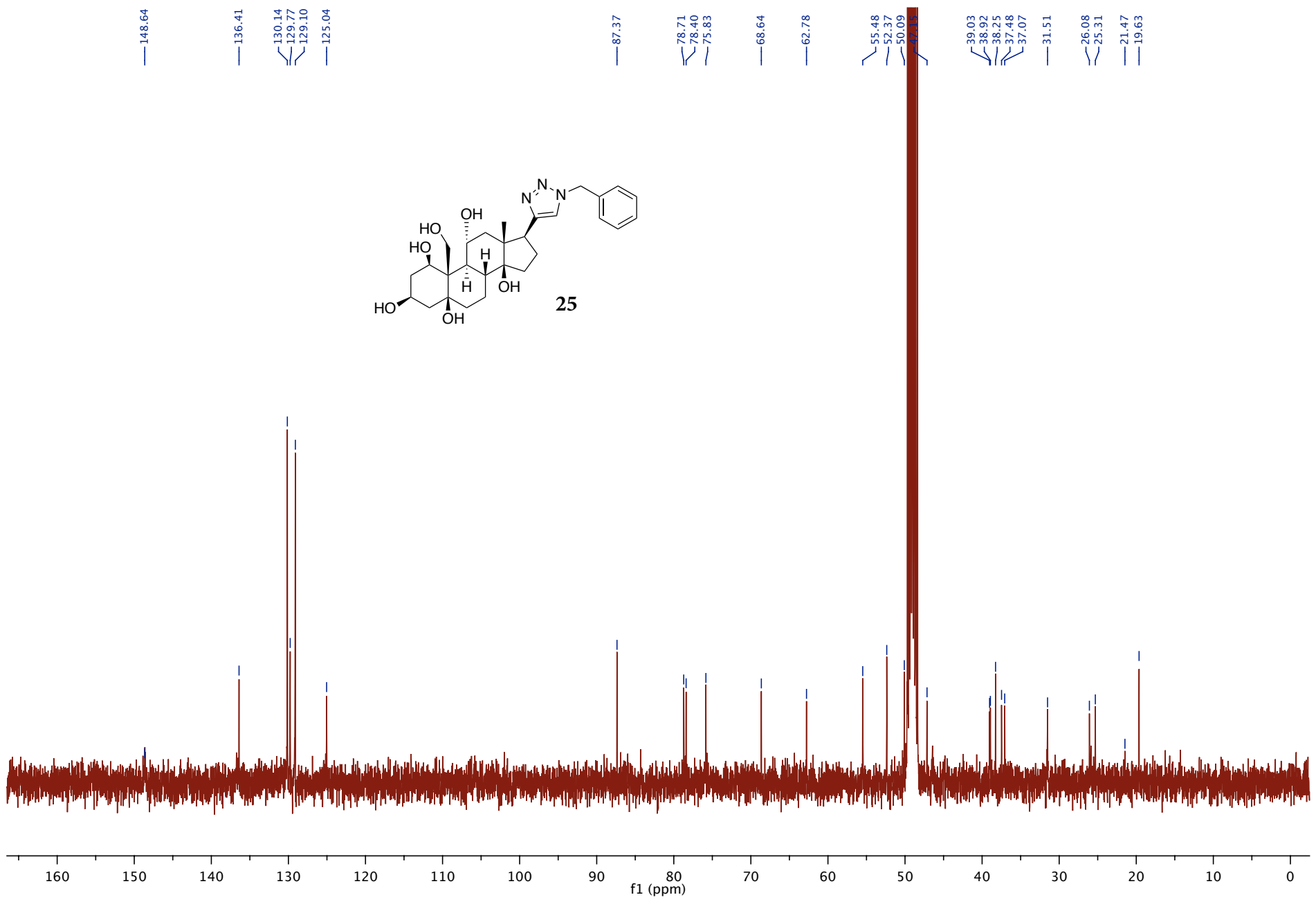
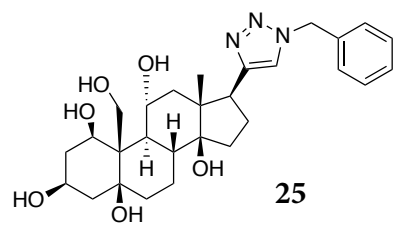


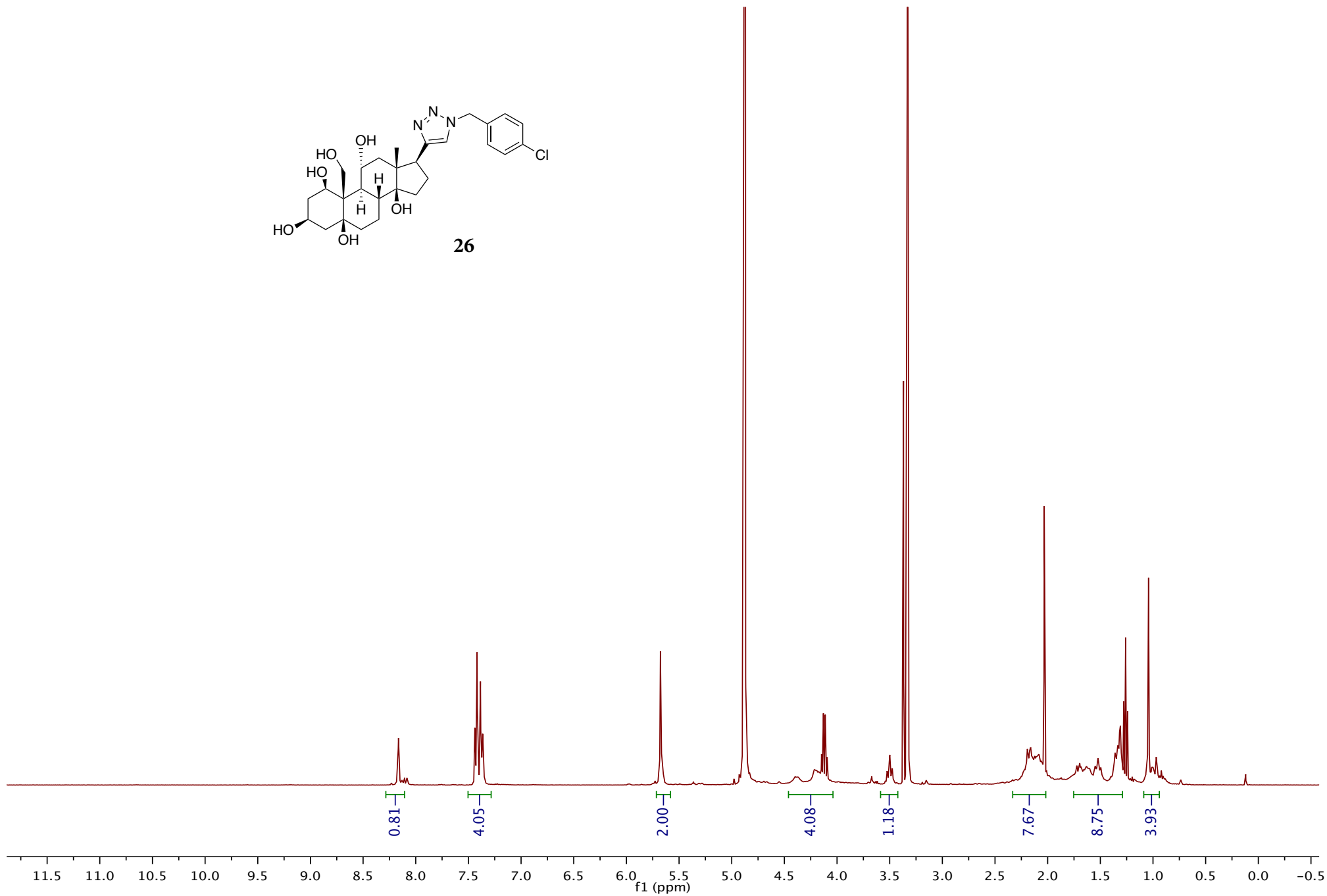
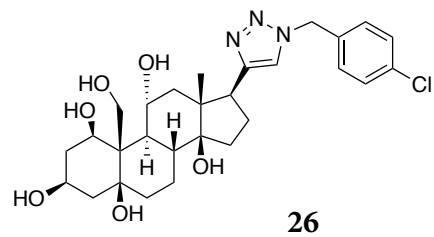


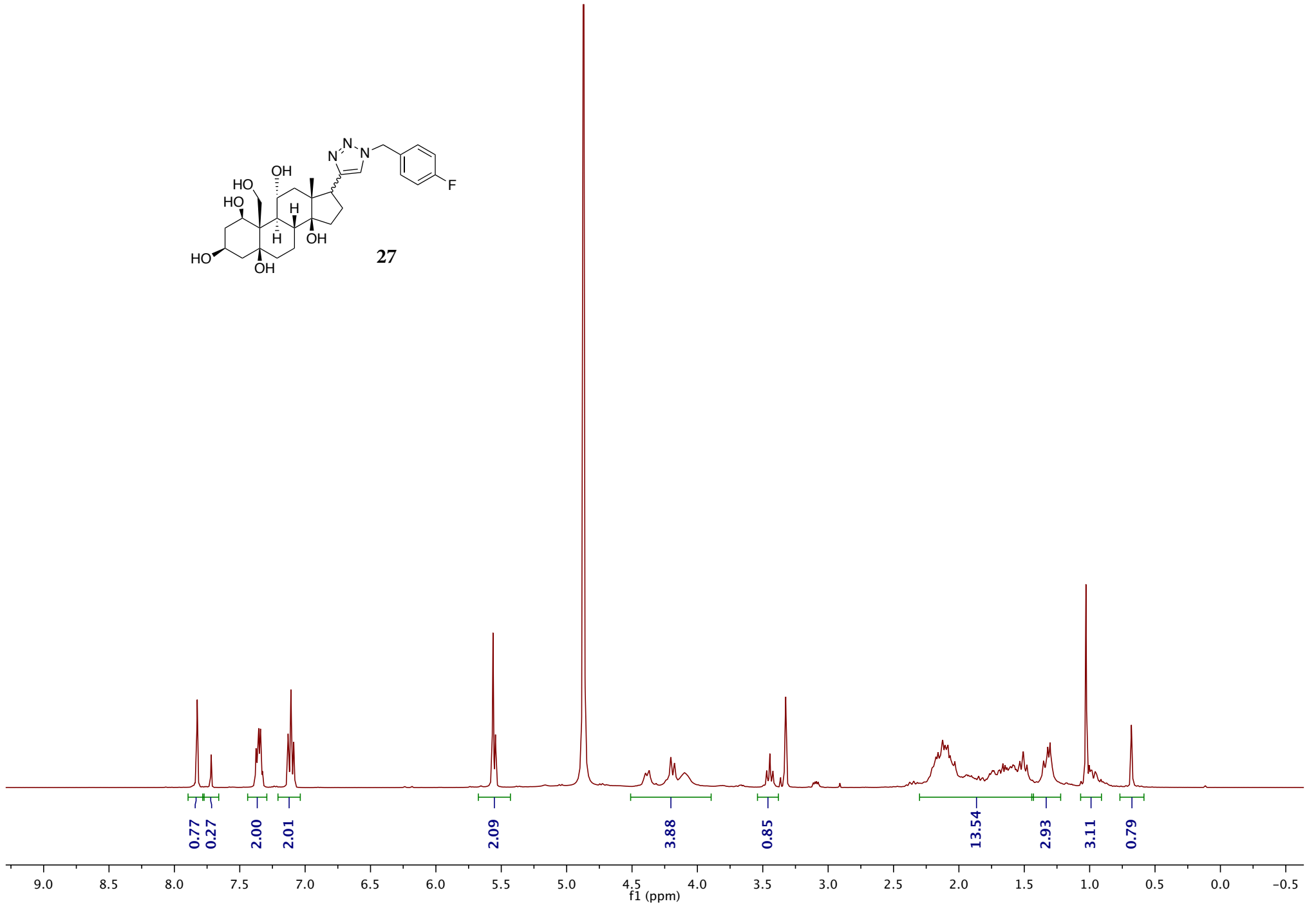
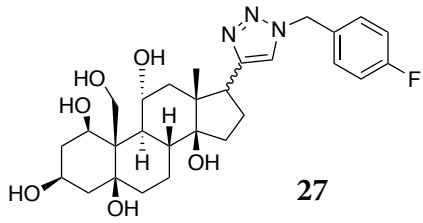
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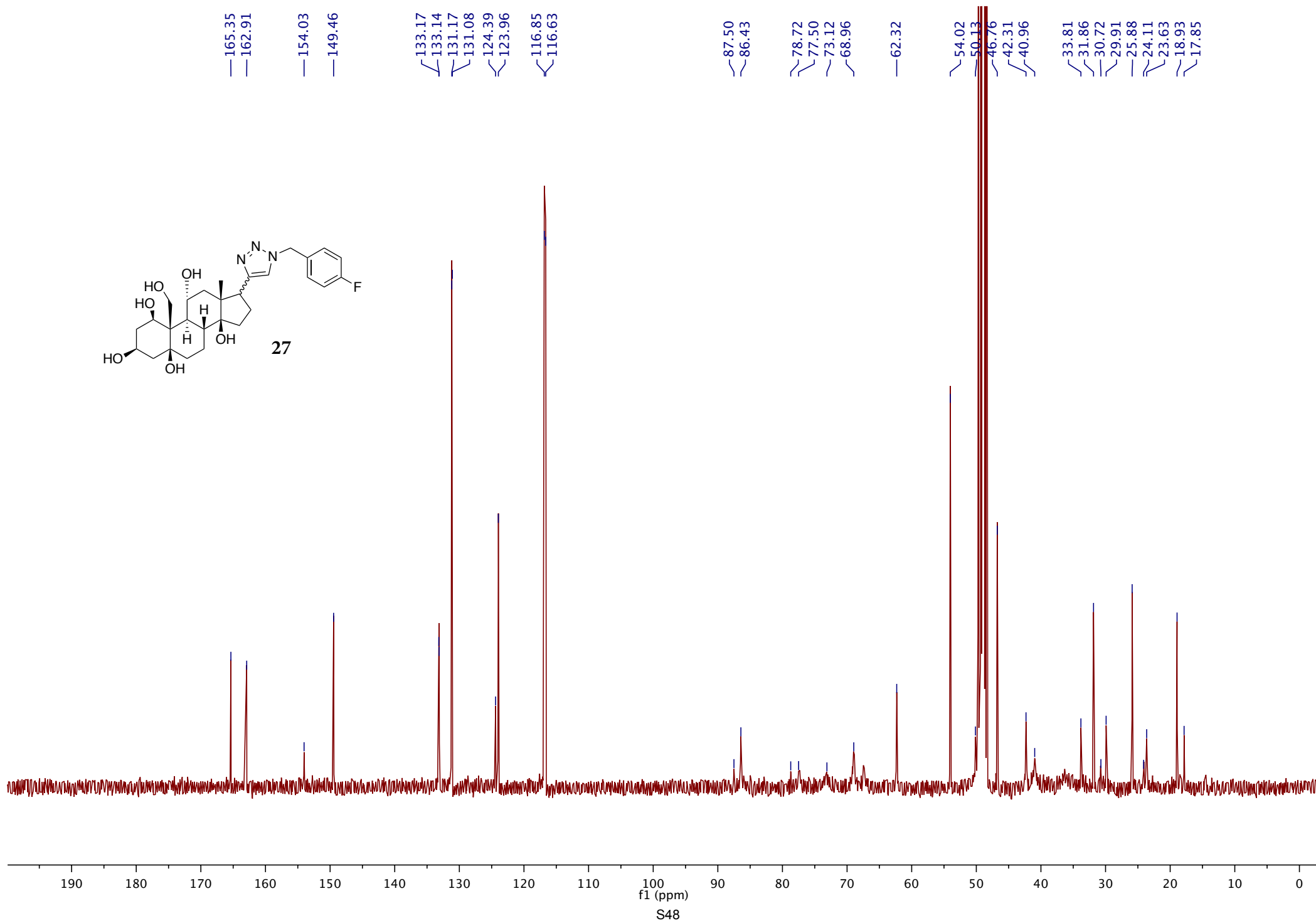
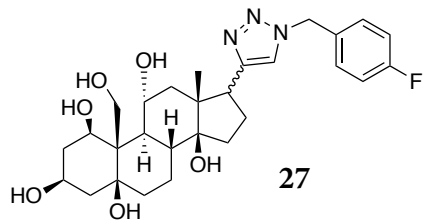




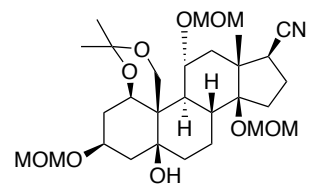




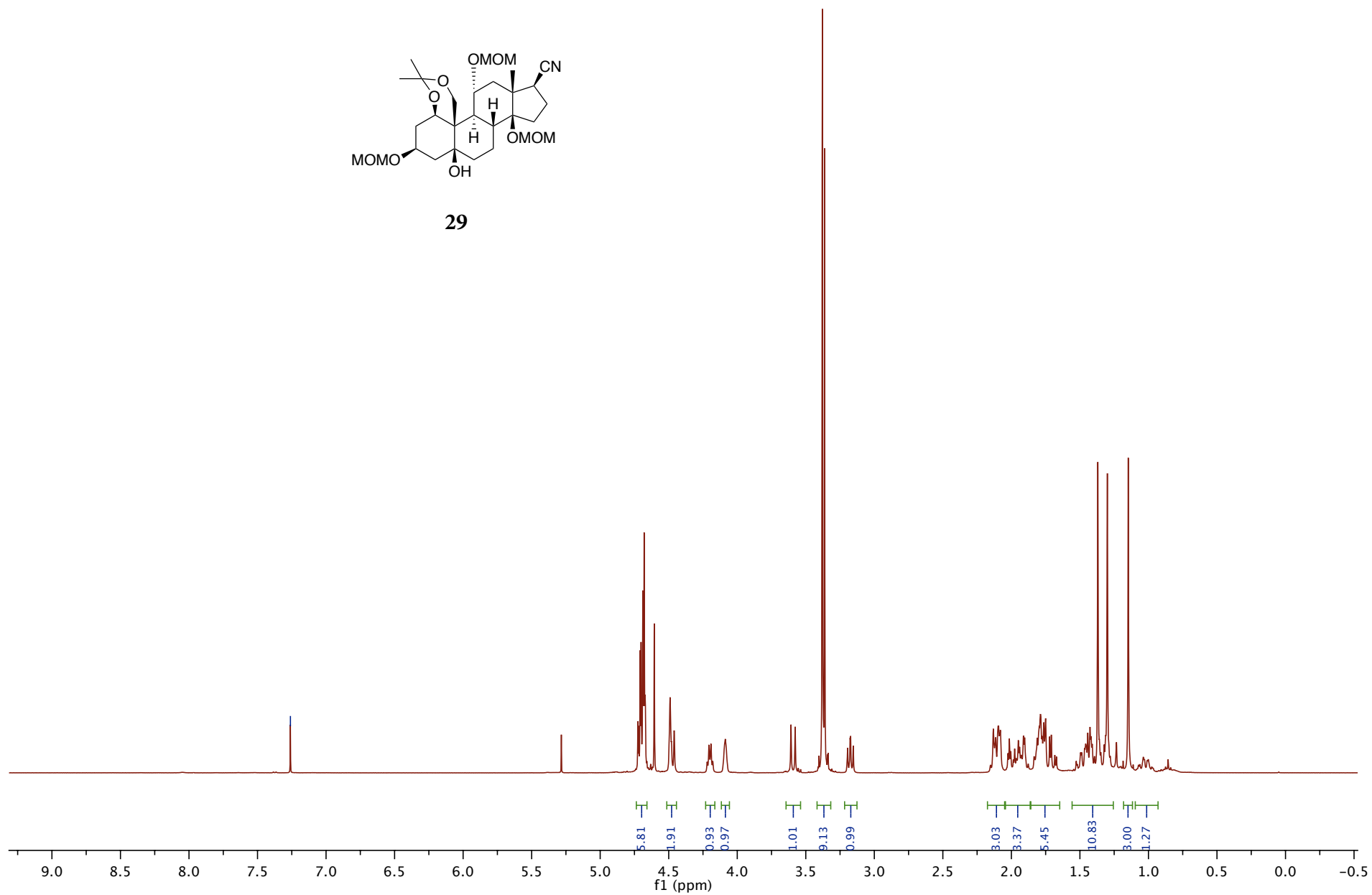


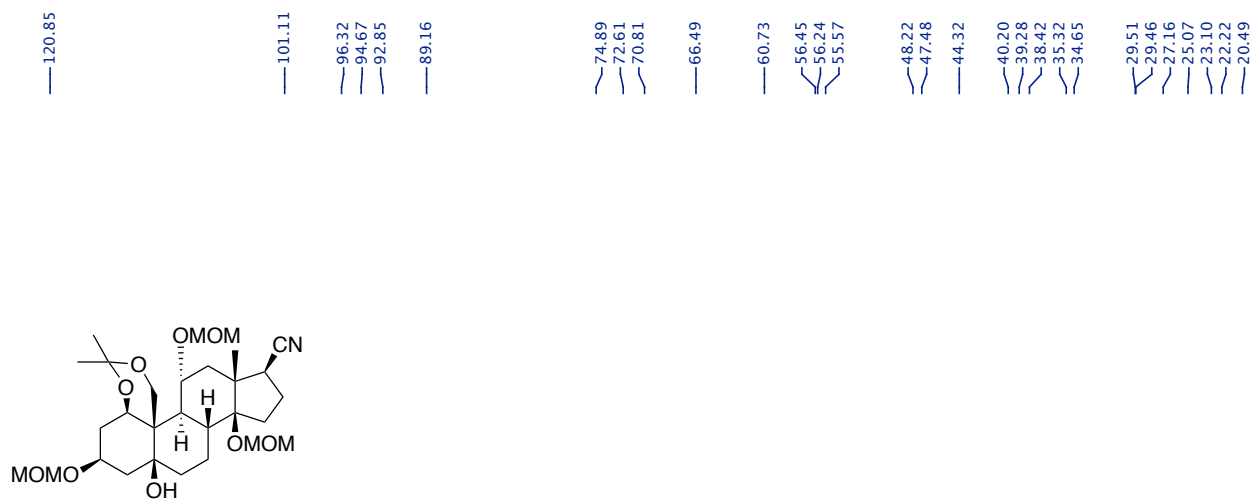


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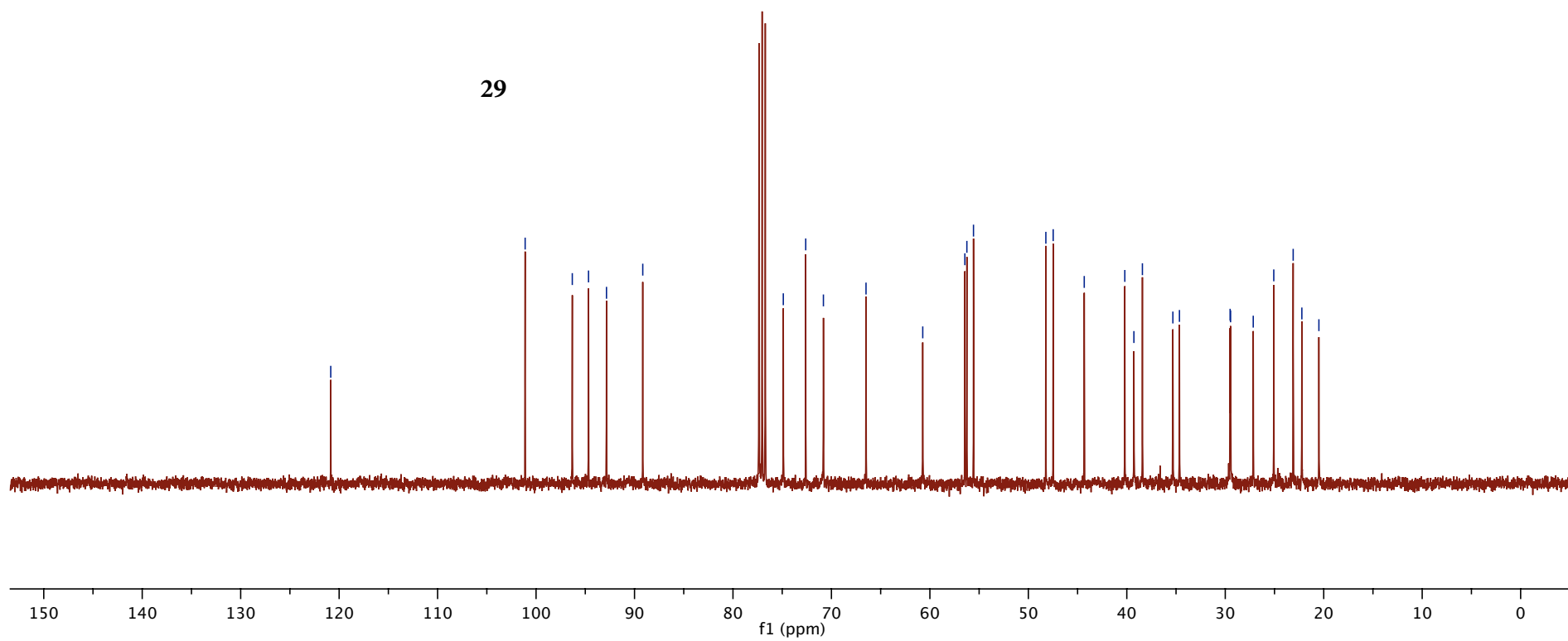


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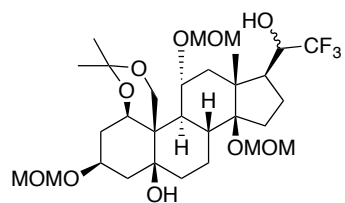




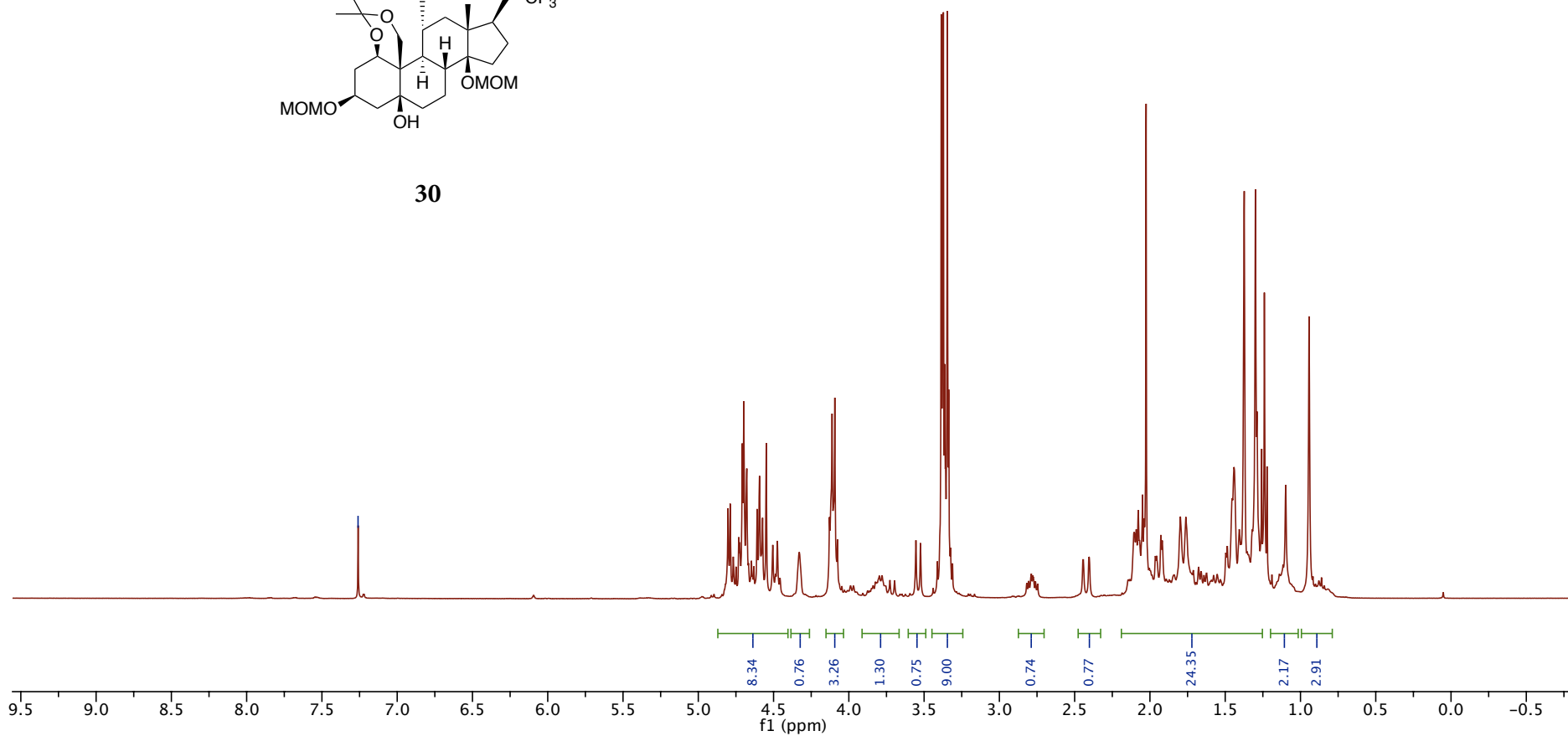
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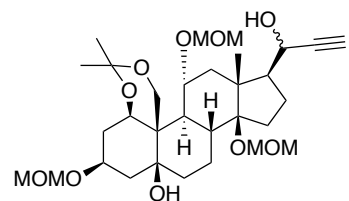


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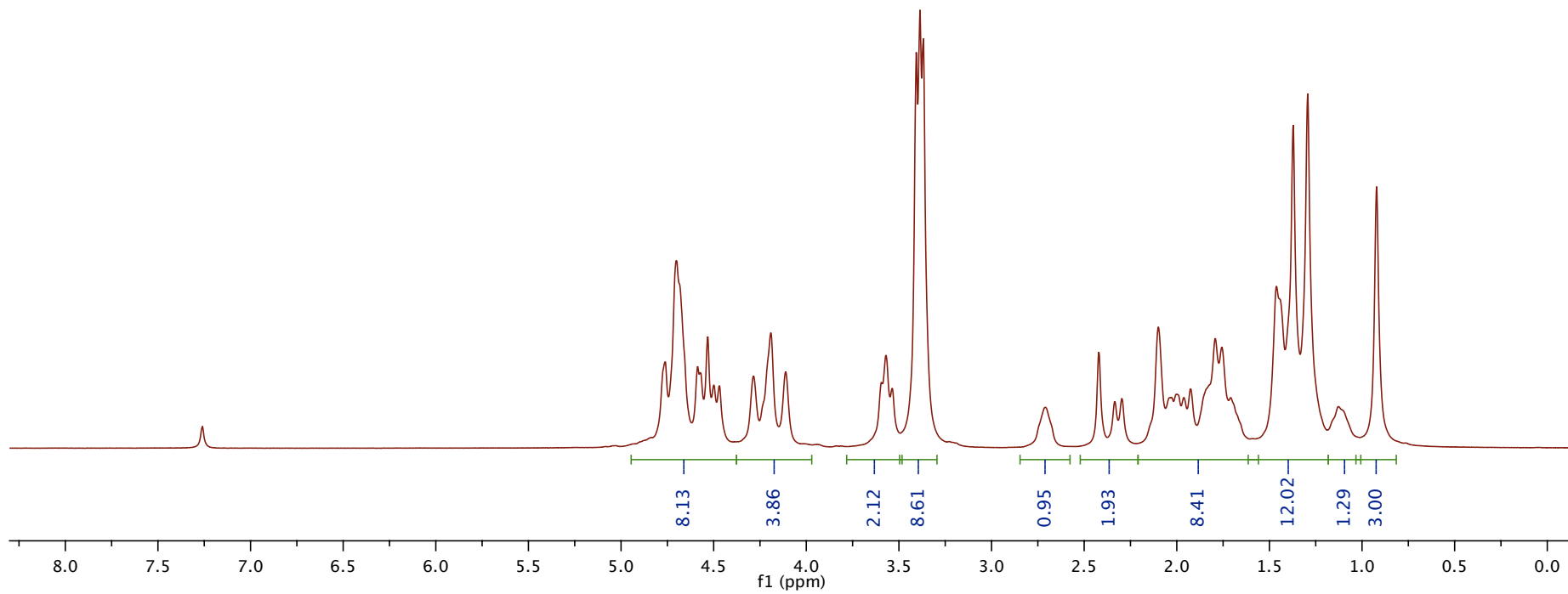


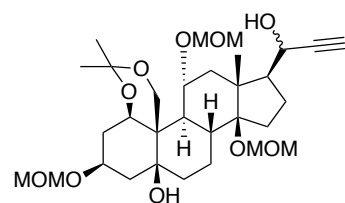
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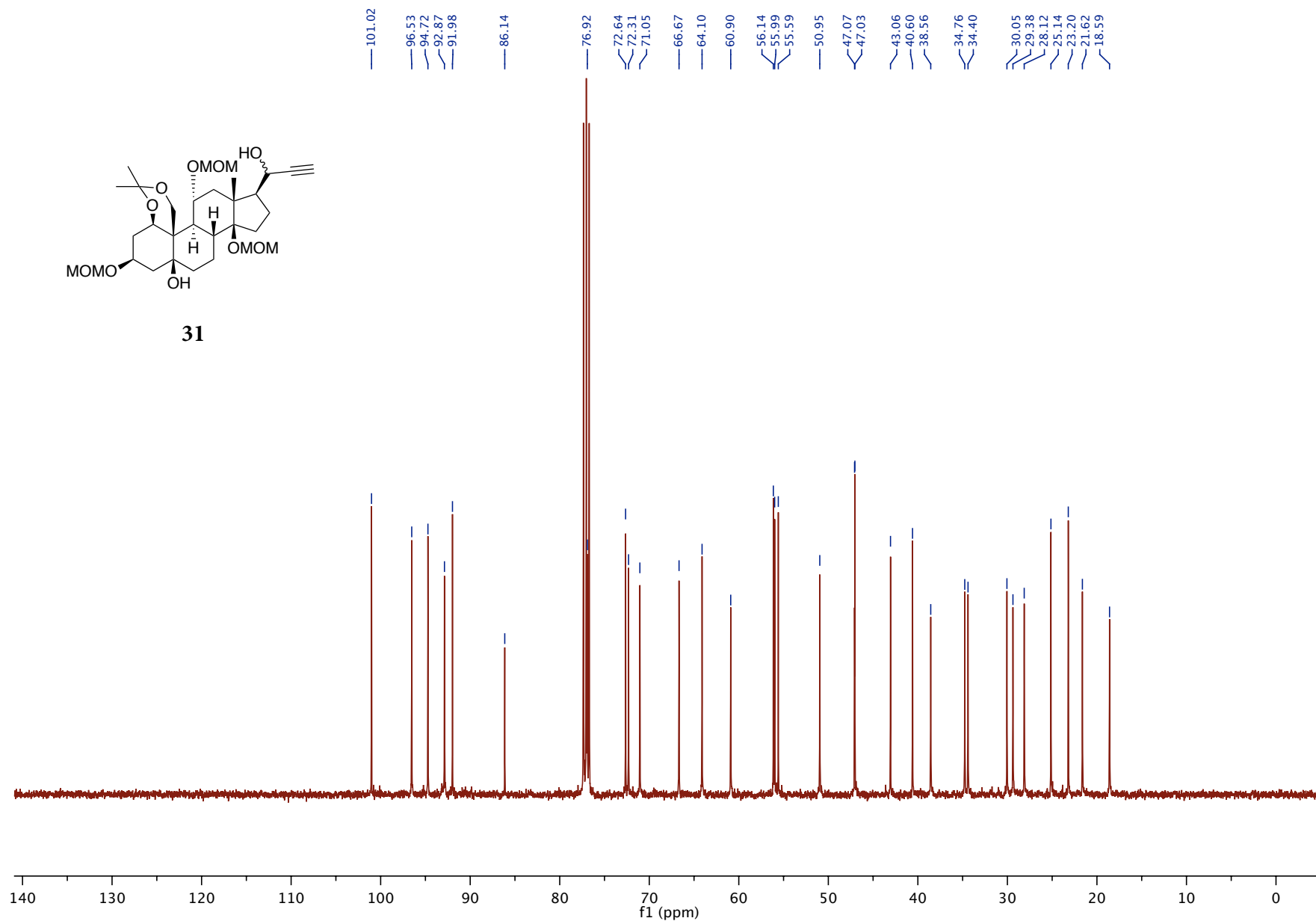


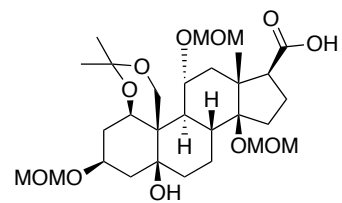
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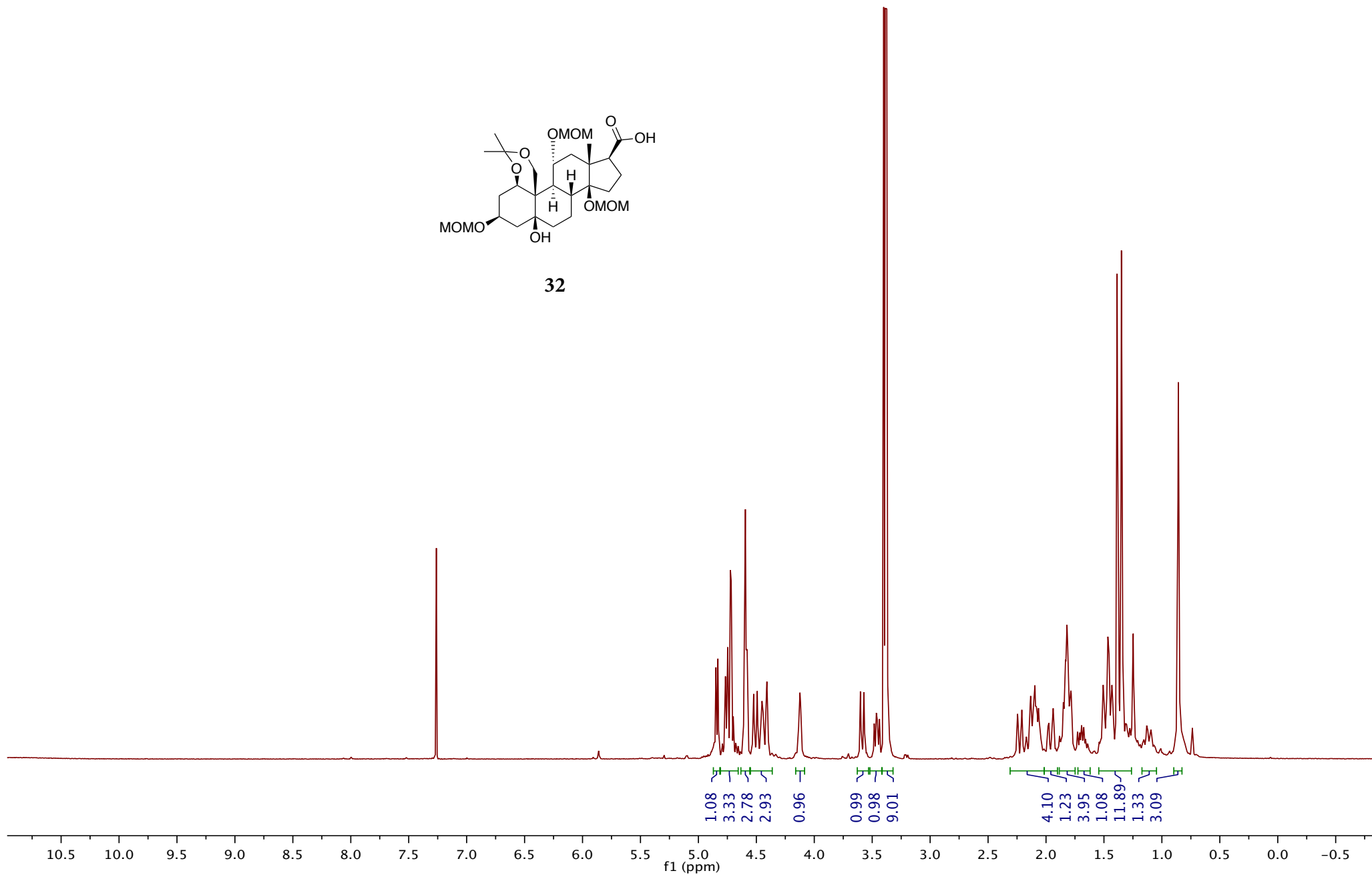


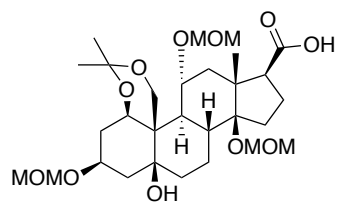
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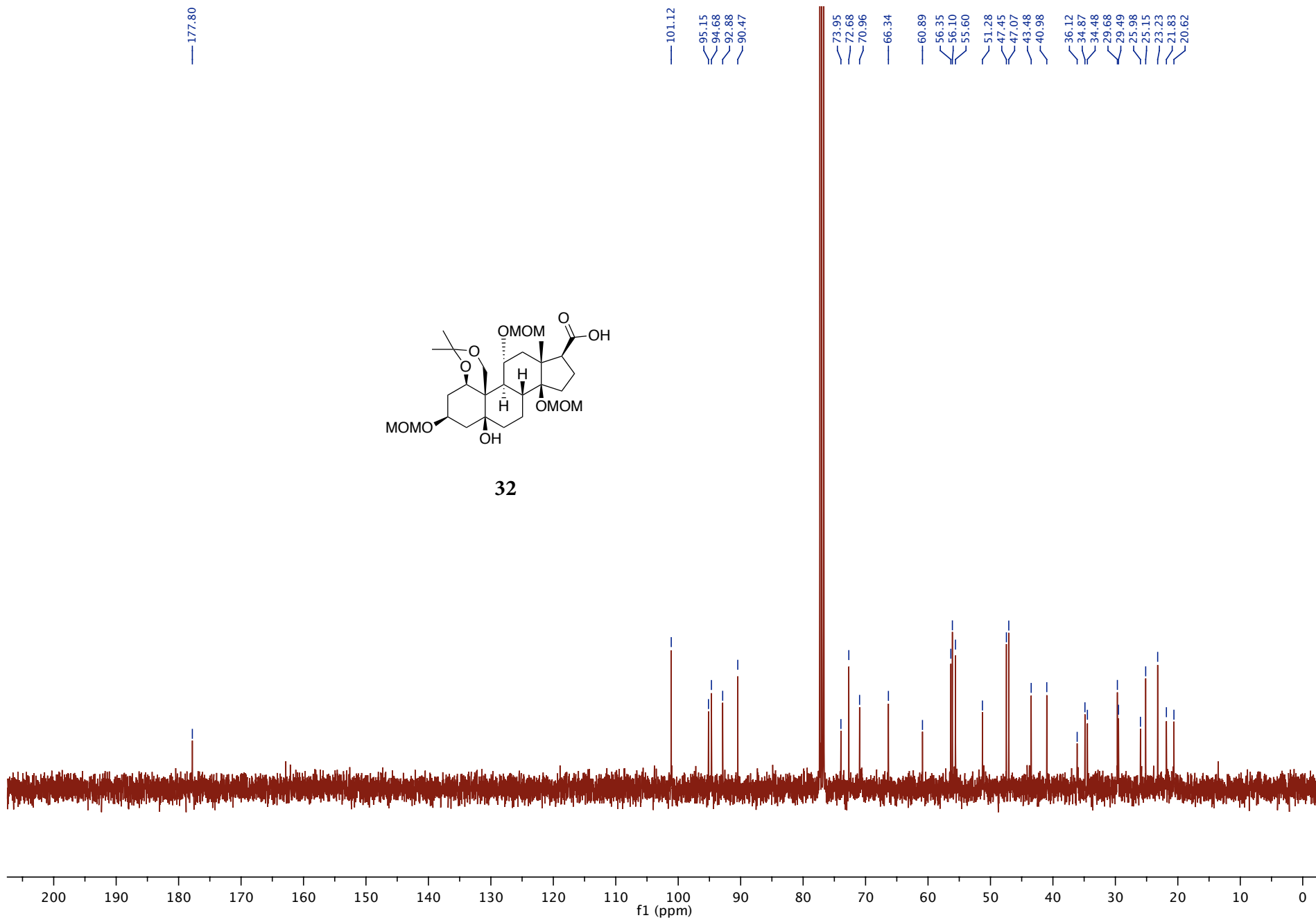


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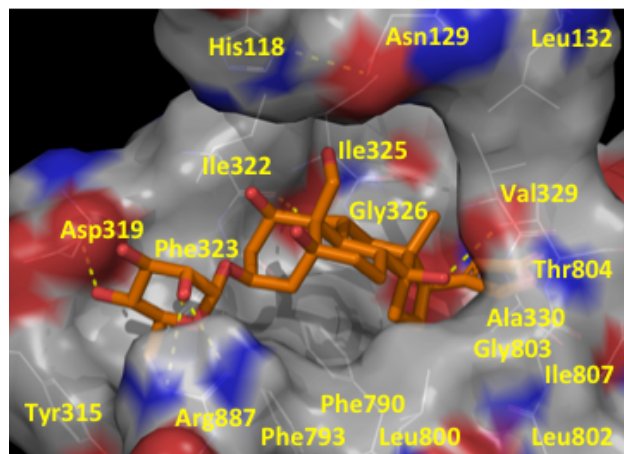




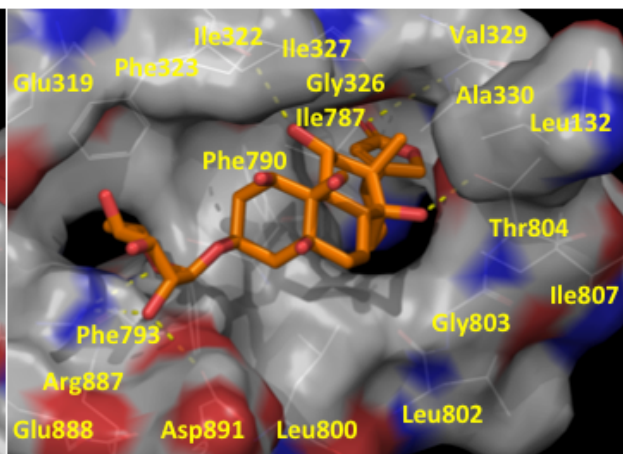
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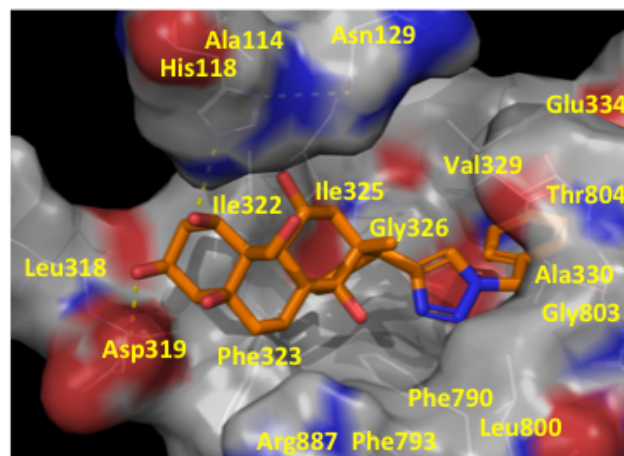
(a)



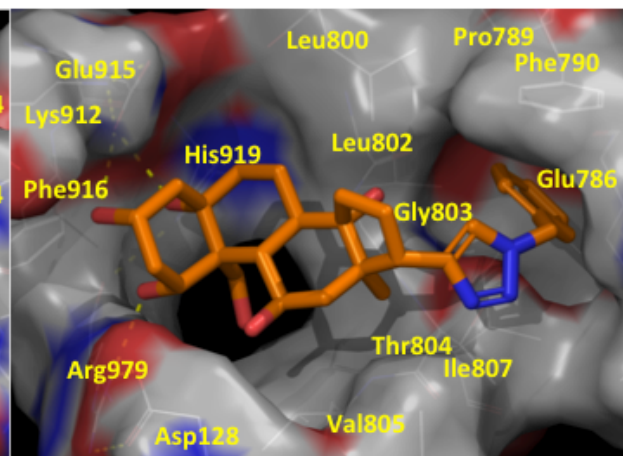
(b)



(c)



(d)



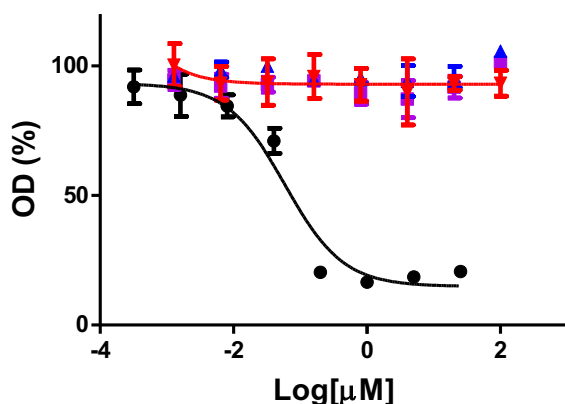
SI Figure 1. Docking poses of ouabain in (a) the rat Na,K-ATPase $\alpha 4$ homology model and (b) the rat Na,K-ATPase $\alpha 1$ homology model, and compound **25** in (c) the rat Na,K-ATPase $\alpha 4$ homology model and (d) the rat Na,K-ATPase $\alpha 1$ homology model.

SI Table 1. Gscores and MM-GBSA relative binding free energies of ouabain and its analogs for the rat Na,K-ATPase $\alpha 1$ and $\alpha 4$ isoforms

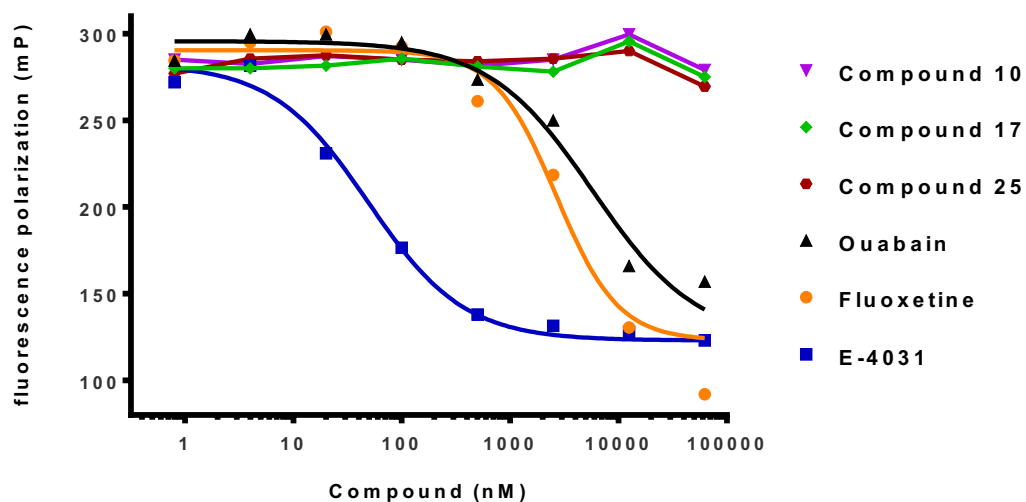
compound	isoform	Gscore	ΔG	IC ₅₀ (M)
ouabain	rat $\alpha 4$	-7.5	-96.3	1.8 x 10 ⁻⁹
	rat $\alpha 1$	-9.8	-92.8	4.3 x 10 ⁻⁵
25	rat $\alpha 4$	-6.7	-74.3	5.3 x 10 ⁻¹²
	rat $\alpha 1$	-6.0	-84.3	5.1 x 10 ⁻⁴

Cancer cell culture and antiproliferation assay

MCF-7 cancer cells were cultured in a T-75 culture flasks with the growth medium RPMI1640 (GIBCO11875) and 10% FBS and incubated at 5% CO₂ and at 37 °C, and subcultured when cells reached 90% confluency. MCF-7 cells were harvested (125X G centrifuge for 5 min) from an exponential-phase maintenance culture. The cells were re-suspended in the fresh culture medium (RPMI1640 (GIBCO11875) with 10% FBS and the cell density was adjusted to 10⁵ cells/mL and dispensed in 96-well culture plates at a density of 5000 cells per 50 µL per well. The cells were incubated overnight to allow cells to adhere to the wells. Assay concentrations were prepared via serial dilution with fresh culture medium with 1% DMSO from 20 mM stock solutions of compounds in DMSO. The various concentrations of the control and test compounds in 50 µL of the culture medium were added to each well. The cultures were incubated for an additional 72 h. Then 10 µL of AlamarBlue was added. Following 1.5 h incubation at 37 °C, the fluorescence, excitation at 530 nm and emission at 590 nm, of each well were measured to determine the optical density. Each compound was tested in triplicate with less than 5% variation.



SI Figure 2. Antiproliferative activity of ouabain (black), and **10** (purple), **17** (blue), and **25** (red) against MCF-7 cells. IC₅₀ of ouabain against MCF-7 is 53±6 nM (47 and 59 nM from two triplicate experiments), but **10**, **17**, and **25** did not exert any growth inhibition of MCF-7 at 100 µM.



SI Figure 3. Potency of cardenolides in the hERG fluorescence polarization assay. Compound displacement of hERG ligand fluorescent tracer from membranes prepared from cells expressing hERG measured by fluorescence polarization according to the manufacturer's instructions (Predictor hERG assay, Invitrogen). Compounds **10**, **17**, and **25** produced negligible inhibition up to a concentration of 62.5 μM . IC_{50} values for ouabain and the hERG reference compounds fluoxetine and E-4031 were 5.8, 2.7, and 0.049 μM , respectively. IC_{50} values were calculated using the fluorescence polarization mP value produced by 62.5 μM E-4031 as bottom plateau to exclude the effect of nonspecific inhibition of binding using Prism 6 (GraphPad). Data is from a single experiment run in duplicate.

SI Table 2. In vitro metabolism stability study: Reference compound data

Assay Reference Compound	Test Concentration (M)	Special Condition	Parent Remaining		
			1 st (%)	2 nd (%)	Mean (%)
Metabolic Stability (liver microsomes, human)					
Imipramine	1.0E-06		84.4	67.9	76
Propranolol	1.0E-06		95.7	92.3	94
Terfenadine	1.0E-06		11.6	8.4	10
Verapamil	1.0E-06		17.2	15.1	16
Metabolic Stability (liver microsomes, monkey, Rhesus)					
Imipramine	1.0E-06	rhesus mnkylm	2.0	1.5	2
Propranolol	1.0E-06	rhesus mnkylm	29.1	26.0	28
Terfenadine	1.0E-06	rhesus mnkylm	1.9	1.5	2
Verapamil	1.0E-06	rhesus mnkylm	1.7	0.6	1
Metabolic Stability (liver microsomes, dog, Beagle)					
Imipramine	1.0E-06		1.4	0.7	1
Imipramine	1.0E-06		1.6	1.3	1
Propranolol	1.0E-06		53.2	44.7	49
Propranolol	1.0E-06		46.9	41.1	44
Terfenadine	1.0E-06		47.5	52.4	50
Verapamil	1.0E-06		14.7	17.4	16
Verapamil	1.0E-06		17.7	15.8	17
Metabolic Stability (liver microsomes, rat, Sprague-Dawley)					
Imipramine	1.0E-06		0.7	0.6	1
Propranolol	1.0E-06		0.0	0.0	0
Terfenadine	1.0E-06		5.4	5.5	5
Verapamil	1.0E-06		27.8	22.3	25
Metabolic Stability (liver microsomes, mouse, CD1)					
Imipramine	1.0E-06		3.5	3.3	3
Propranolol	1.0E-06		24.7	24.1	24
Terfenadine	1.0E-06		8.8	6.4	8
Verapamil	1.0E-06		7.5	6.0	7

SI Table 3. In vitro absorption assays: Reference compound data

Assay Reference Compound	Test Concentration (M)	Permeability			Flags	Percent Recovery		
		1 st (10 ⁻⁶ cm/s)	2 nd (10 ⁻⁶ cm/s)	Mean (10 ⁻⁶ cm/s)		1 st (%)	2 nd (%)	Mean (%)
A-B Permeability (Caco-2, pH 6.5/7.4)								
Colchicine	1.0E-05	0.08	0.08	<0.1	BLQ	93	92	93
Labetalol	1.0E-05	5.63	5.95	5.8		81	87	84
Propranolol	1.0E-05	25.85	28.30	27.1		65	66	65
Ranitidine	1.0E-05	0.23	0.16	0.2		98	95	97
B-A Permeability (Caco-2, pH 6.5/7.4)								
Colchicine	1.0E-05	8.61	9.43	9.0		98	92	95
Labetalol	1.0E-05	42.07	41.15	41.6		91	94	93
Propranolol	1.0E-05	30.90	32.44	31.7		92	93	92
Ranitidine	1.0E-05	5.12	4.03	4.6		107	105	106

BLQ Below the Limit of Quantitation. Test compound was well detected in donor samples but not detected in receiver samples. The concentration of test compound in receiver sample was below the limit of quantitation.

ABBREVIATIONS USED:

Gscores (Glide score); MMGBSA (molecular mechanics energies generalized Born and surface area continuum solvation method).