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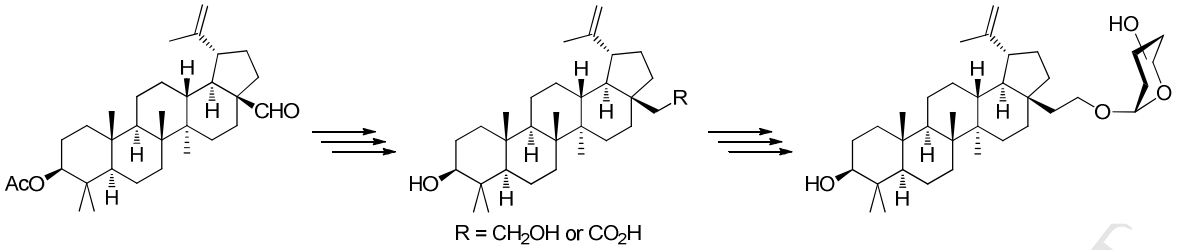
Graphical abstract



Synthesis and biological activity of new homolupanes and homolupane saponins.

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Abstract: A concise synthesis of 28a-homolupane triterpenes and the corresponding saponins containing D-mannose, D-idose, D-arabinose and L-rhamnose moieties was elaborated. The overall synthesis of the new triterpenes involved three linear steps starting from readily available 3-*O*-acetyl-betulinal: elongation of the carbon chain by Wittig reaction followed by enol ether hydrolysis and reduction (or oxidation) of the elongated aldehyde. Saponins were obtained by glycosylation of triterpenes with classical Schmidt donors.

Cytotoxic activities of new lupane and homolupane compounds were evaluated *in vitro*. Several triterpenes and the corresponding saponins exhibited an interesting cytotoxic activity profile against human cancer cell lines. Influence of the side-chain structure and substituents on the cytotoxicity of betulin and homobetulin derivatives was investigated. These results open the way to the synthesis of various lupane-type triterpene and saponin derivatives as potential anticancer compounds.

Keywords: Homobetulin; Homobetulinic acid; Glycosylation; Lupane saponins; Homolupane saponins; SAR study.

1. Introduction

Betulin (**1**) and betulinic acid (**2**) are natural compounds with proven anticancer, antibacterial, antimalarial, antiviral and antiinflammatory activities.[1-6] These triterpene class compounds have attracted high interest due to their strong biological activities and low toxicity.[7-10] Recent investigations have demonstrated that even a simple modification of betulin or betulinic acid can profoundly influence their anticancer and anti-HIV activities.[11-13] The best example is Bevirimat (**3**), betulinic acid modified at the C-3

hydroxyl group, a potent anti-HIV agent that blocks HIV-1 replication, which is currently in phase IIb clinical trials (Figure 1).[14-16]

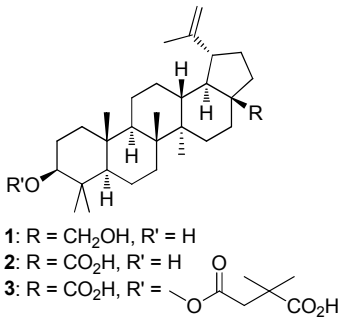


Figure 1. Structures of betulin (**1**), betulinic acid (**2**), and Bevirimat (**3**).

We are particularly interested in the chemistry and biological roles of lupane saponins. Saponins are steroid or triterpenoid glycosides, widely distributed in plants and some marine organisms, which have interesting biological properties.[17,18] Natural saponins based on the betulin scaffold occur less frequently than those with other triterpene-type aglycones, but interest in their synthesis is growing.[19-23] However, despite strenuous efforts, no lupane derivatives that could be used as anticancer drugs have been found to date, generally because they have too low cytotoxicity.[24,25] Therefore, identifying new betulin derivatives with high cytotoxic activity and potential as drug candidates is still a major challenge.

Early investigations demonstrated that even simple modifications at C-3 and/or C-28 positions of betulin and betulinic acid significantly change the cytotoxic activity of the resulting compounds.[25-30] However, little information is available on the effects of substitution at the lupane C-28 carbon and cytotoxicity of the resulting saponins.[2-4,11,27] In addition, there are gaps in the knowledge of the structure-activity relationships (SAR) of variations in the branching at the C-28 position.

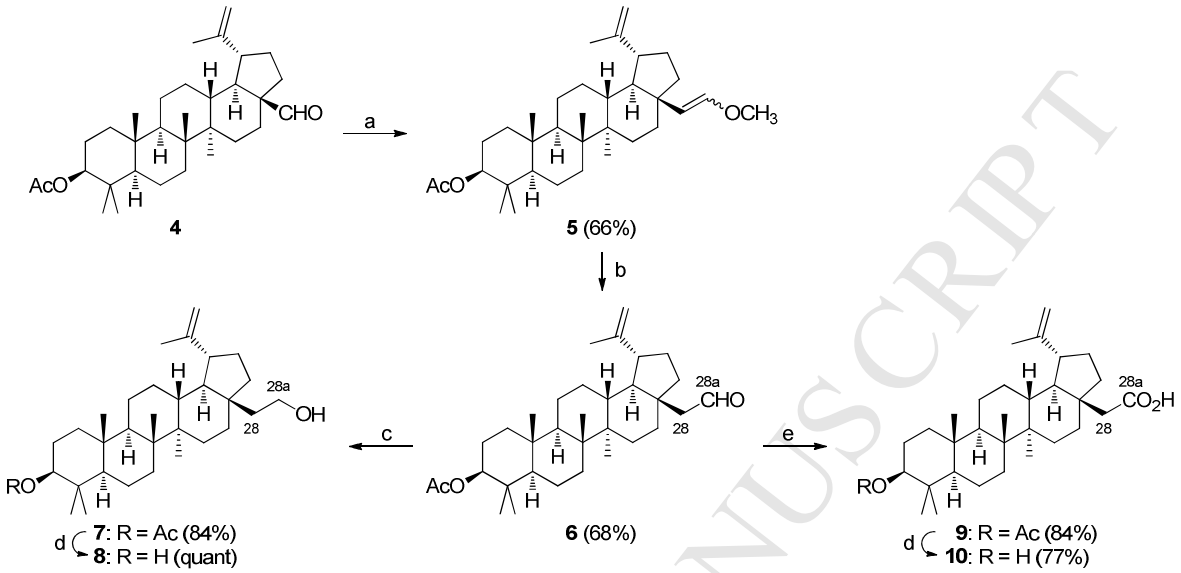
Herein we report the synthesis of novel homolupane triterpenes *via* elongation of the side-chain at the lupeol C-17 position by Wittig-type reaction and transformation of the products into saponins, and tests of their cytotoxicity against a series of cell lines. We also discuss effects of the substituents and elongation of the side-chain on cell cytotoxicity.

2. Results and discussion

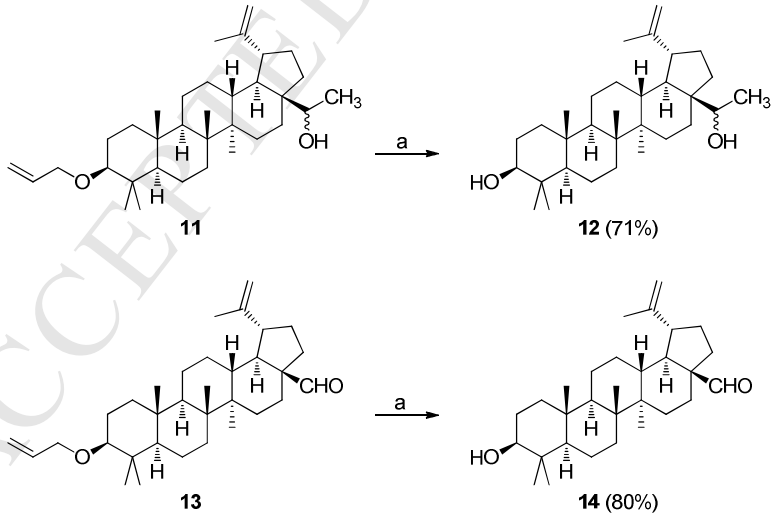
2.1. Synthesis of homolupanes

The synthesis of homobetulin (ichopanol, **8**) and homobetulinic acid (ichopanic acid, **10**) is presented in Scheme 1. The Wittig reaction of easily available aldehyde **4**[27] with a (methoxymethyl)triphenylphosphorane [obtained from (methoxymethyl)triphenylphosphonium chloride by treatment with *n*-butyllithium] gave enol ether **5** as a mixture of *(E)*- and *(Z)*-isomers in 66% yield. Enol ether **5** was hydrolytically stable and its transformation into aldehyde **6** by treatment with PPTS in the acetone/water mixture required a long reaction time (48 h) and elevated temperature (60 °C). The expected elongated aldehyde **6** was obtained in 68% yield.

Further reduction of **6** with $NaBH_4$ gave homobetulin acetate **7** in 84% yield. Deacetylation by treatment with refluxing ethanolic KOH solution afforded ichopanol (**8**) quantitatively. Oxidation of aldehyde **6** by treatment with Jones reagent at 0 °C in acetone provided the corresponding acid **9** in 84% yield. Deacetylation of **9**, as described for alcohol **8**, gave the free ichopanic acid (**10**) in 77% yield.



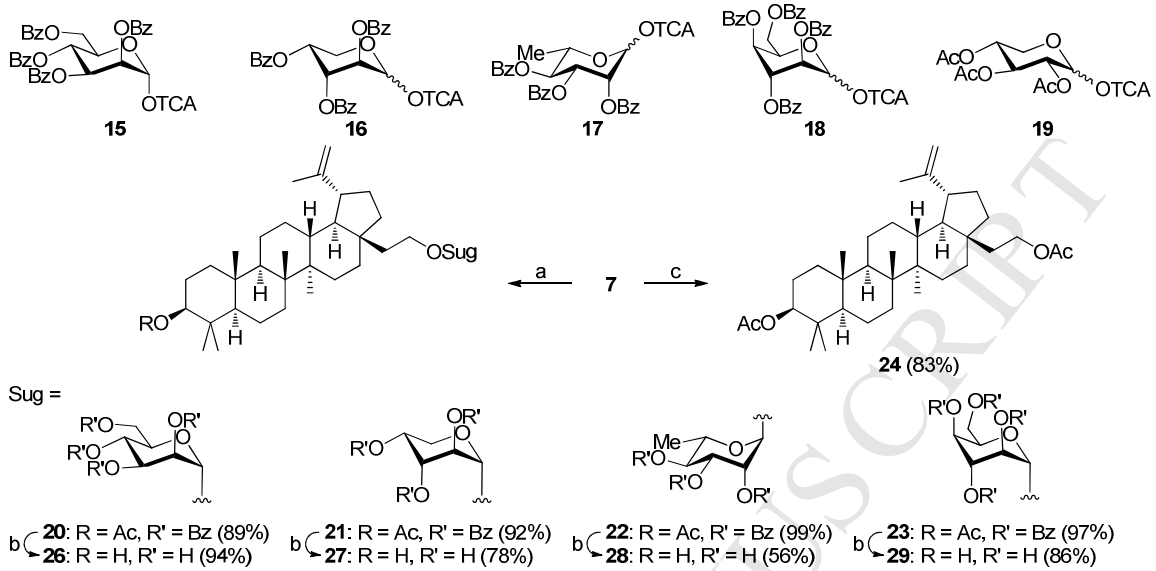
Scheme 1. Reagents and conditions: (a) (methoxymethyl)triphenylphosphonium chloride (5 equiv.), n-butyllithium (4 equiv.), 0 °C; (b) PPTS, acetone/water, 60 °C; (c) $NaBH_4$, CH_3OH; (d) KOH (2.5 equiv.), ethanol, reflux; (e) Jones reagent, acetone.



Scheme 2. Reagents and conditions: (a) $PdCl_2$, MeOH, DCM.

Isomeric homobetulin **12**, in which the hydroxyl group is connected to the C-28 atom was obtained in 71% yield by palladium(II) chloride-induced deallylation of alcohol **11**[27] (Scheme 2). Betulinal (**14**) was prepared in 80% yield by similar deallylation of aldehyde **13**[27] (Scheme 2).

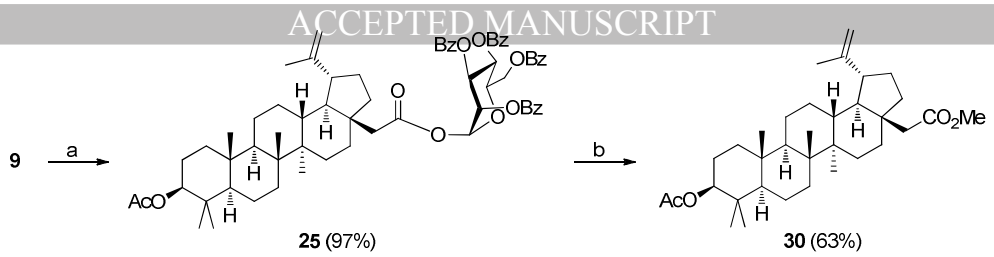
2.2. Synthesis of saponins



Scheme 3. Reagents and conditions: (a) **15**–**18**, CH_2Cl_2, TMSOTf, –40 °C; (b) K_2CO_3, methanol; (c) **19**, CH_2Cl_2, TMSOTf, –40 °C.

Ichopanol acetate (**7**) was glycosylated by treatment with perbenzoylated glycosyl donors **15**,[31] **16**,[28] **17**,[28] and **18**[32] in the presence of TMSOTf under standard conditions (Scheme 3).[26] The glycosides **20**-**23** were obtained in excellent yields ranging from 89% to 99%. As expected, the presence of benzoyl protecting groups in position 2 of the sugar donors directed anomeric selectivity of the glycosidation reaction.[33] In all cases 1,2-*trans*-monodesmosidic saponins (α-D-mannopyranosides, α-D-arabinopyranosides, α-L-rhamnopyranosides, and α-D-idopyranosides) were obtained exclusively, as confirmed by the chemical shifts and vicinal coupling constants of the anomeric protons. Final deprotection of the hydroxyl groups was performed by treating protected saponins with potassium carbonate in methanol. Under the applied reaction conditions, deacetylation at the O-3 position of the lupane core was also observed. This contrasts with our earlier observations that acetyl groups in lupanes are hydrolytically stable.[26,34] Free saponins **26**-**29** were obtained in good yields (56-94%).

By comparison, attempt to glycosylation of ichopanol acetate (**7**) with 2,3,4-tri-*O*-acetyl-D-xylopyranosyl trichloroacetimidate (**19**)[35] under standard conditions completely failed, and diacetate **24** was obtained as the only product in high yield (83%).



Scheme 4. Reagents and conditions: (a) **15**, TMSOTf, CH_2Cl_2, –40 °C; (b) K_2CO_3, CH_3OH.

Glycosylation of ichopanic acid acetate (**9**) with 2,3,4,6-tetra-*O*-benzoyl-α-D-mannopyranosyl trichloroacetimidate (**15**) gave the expected glycosyl ester **25** in 97% yield. Its treatment with potassium carbonate in methanol gave methyl ester **30** as the only product in good yield (63%). When sodium methoxide in methanol was used, decomposition of the starting material occurred (Scheme 4).

The structures of all new compounds were confirmed by extended 1D and 2D NMR experiments (Tables 1 and 2), as well as elemental analysis and HRMS. In addition, the crystal structure of homobetulin (**8**) was unequivocally confirmed by X-ray analysis (Figure 2).

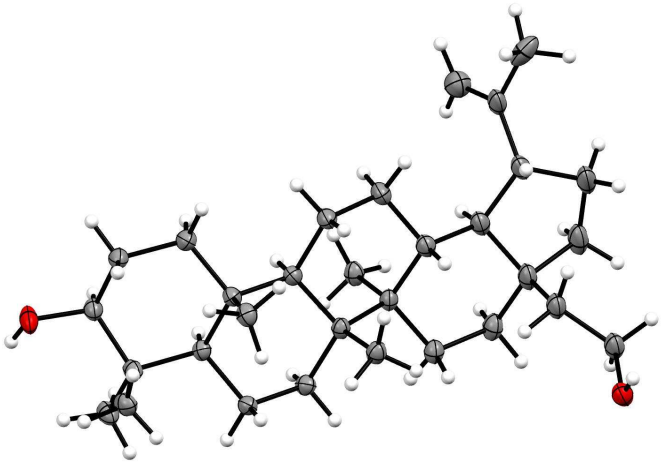


Figure 2. X-Ray structure of **8**.

2.3. Results of *in vitro* anticancer activity assays

Anticancer activities of the studied homolupane triterpenes and saponins with a modified side-chain at the C-17 position were tested *in vitro* and compared with activities of the structurally similar compounds we have previously described.[26,27] Several normal and cancer cell lines were cultured and used to examine structure-activity relationships of these lupane triterpenes. We compared the *in vitro* cytotoxic activity of selected analogues against human BJ fibroblasts and cancer cell lines of various histopathological origins, including T-lymphoblastic leukemia CEM, breast adenocarcinoma MCF7, and cervical carcinoma HeLa lines. Cells of all of these lines were exposed to six serial 3-fold dilutions of each drug for 72 h, the proportions of surviving cells were then estimated and IC_{50} values (50% inhibitory concentrations) were calculated. The results obtained from Calcein AM assays are presented in Table 3.

Table 1. [1]H NMR spectroscopic data for compounds **7**-**10**, **20**-**23,** and **25**-**29**.

Atom No.	**7** [a,b]	**8** [a]	**9** [a,b]	**10** [a]	**20** [a,b]	**21** [a,b]	**22** [a,b]
triterpene unit:							
1	1.00, 1.67	0.91, 1.67	0.98, 1.66	0.90, 1.66	1.00, 1.68	0.96, 1.64	1.0, 1.68
2	1.62	1.38, 1.61	1.61	1.58	1.63	1.59	1.59, 1.62
3	4.48	3.19	4.47	3.19	4.48	4.46	4.48
5	0.79	0.68	0.79	0.69	0.80	0.75	0.85
6	1.40, 1.57	1.40, 1.52	1.40, 1.51	1.40, 1.53	1.40, 1.51	1.32, 1.46	1.39, 1.51
7	1.40	1.38	1.40	1.40	1.41	1.29	1.42
9	1.31	1.28	1.30	1.28	1.32	1.23, 1.24	1.33
11	1.23, 1.43	1.23, 1.43	1.22, 1.42	1.23, 1.43	1.27, 1.44	1.15, 1.38	1.28, 1.45
12	1.07, 1.66	1.06, 1.65	1.08, 1.64	1.07, 1.64	1.06, 1.69	1.01, 1.58	1.10, 1.70
13	1.78	1.78	1.79	1.61	1.83	1.65	1.84
15	1.01, 1.65	1.01, 1.66	1.06, 1.75	1.06, 1.76	1.04, 1.69	0.82, 1.50	1.08, 1.69
16	1.22, 1.64	1.22, 1.64	1.32, 1.64	1.32, 2.00	1.25, 1.66	1.11, 1.57	1.44, 1.92
18	1.42	1.42	1.54	1.54	1.50	1.36	1.48
19	2.42	2.42	2.35	2.35	2.48	2.28	2.48
21	1.36, 1.93	1.36, 1.93	1.41, 2.00	1.40, 2.00	1.39, 1.96	1.30, 1.85	1.41, 1.99
22	1.05, 1.65	1.04, 1.64	1.16, 1.95	1.16, 1.94	1.25, 1.64	0.97, 1.59	1.28, 1.70
23	0.85	0.76	0.84	0.76	0.84	0.84	0.83
24	0.83	0.97	0.85	0.96	0.85	0.85	0.84
25	0.86	0.83	0.86	0.83	0.87	0.81	0.87
26	1.04	1.05	1.04	1.04	1.10	0.83	1.11
27	0.96	0.96	0.97	0.97	0.97	0.87	0.99
28	1.28, 1.81	1.28, 1.82	0.90, 2.04	2.04, 2.53	1.41, 1.97	1.28, 1.80	1.44, 1.92
28a	3.70-3.63	3.70, 3.64	–	–	3.58, 3.85	3.54, 3.94	3.57, 3.84
29	4.58, 4.69	4.58, 4.69	4.59, 4.69	4.58, 4.68	4.61, 4.74	4.55, 4.63	4.56, 4.68
30	1.68	1.68	1.69	1.69	1.71	1.64	1.71
monosaccharide unit:							
1	–	–	–	–	5.09	4.74	5.01
2	–	–	–	–	5.69	5.73	5.64
3	–	–	–	–	5.93	5.59	5.84
4	–	–	–	–	6.07	5.69	5.67
5	–	–	–	–	4.42	3.91, 4.34	4.19
6	–	–	–	–	4.51, 4.69	–	1.37

[a]Recorded in $CDCl_3$ at 600 MHz, δ in ppm. [b]3-*O*-Acetyl group: OCH_3 2.04 (s) ppm. [c]Recorded in $CDCl_3$ / CD_3OD mixture (1:1) at 600 MHz, δ in ppm.

Table 1 contd. ^{1}H NMR spectroscopic data for compounds **7**-**10**, **20**-**23,** and **25**-**29**.

Atom No.	**23** [a,b]	**25** [a,b]	**26** [c]	**27** [c]	**28** [c]	**29** [c]
triterpene unit:						
1	0.97, 1.66	1.01, 1.69	0.92, 1.68	0.90, 1.67	0.91, 1.67	0.92, 1.68
2	1.59, 1.62	1.62	1.56, 1.61	1.57	1.58	1.59
3	4.46	4.53-4.43	3.12	3.17	3.17	3.16
5	0.77	0.82	0.70	0.69	0.68	0.69
6	1.34, 1.48	1.43, 1.51	1.43, 1.54	1.39, 1.52	1.40, 1.52	1.40, 1.53
7	1.32	1.46	1.42	1.38	1.39, 1.66	1.40
9	1.27	1.34	1.34	1.28	1.28	1.30
11	1.18, 1.41	1.28, 1.45	1.26, 1.44	1.22, 1.43	1.23, 1.43	1.24, 1.44
12	1.05, 1.64	1.12, 1.70	1.10, 1.69	1.06, 1.65	1.06, 1.66	1.08, 1.67
13	1.70	1.69	1.82	1.77	1.76	1.77
15	0.91, 1.53	1.17, 1.85	1.07, 1.72	1.01, 1.62	0.96, 1.62	0.97, 1.63
16	1.16, 1.54	1.38, 2.03	1.25, 1.73	1.22, 1.62	1.14, 1.72	1.16, 1.54
18	1.40	1.58	1.48	1.44	1.44	1.46
19	2.38	2.44	2.42	2.40	2.40	2.40
21	1.28, 1.89	1.47, 2.06	1.35, 1.94	1.35, 1.91	1.35, 1.91	1.37, 1.91
22	0.94, 1.58	1.22, 1.94	1.05, 1.73	1.04, 1.64	1.03, 1.64	1.03, 1.64
23	0.83	0.85	0.75	0.76	0.76	0.76
24	0.84	0.86	0.94	0.96	0.96	0.96
25	0.82	0.89	0.86	0.83	0.83	0.85
26	0.88	1.13	1.08	1.03	1.04	1.05
27	0.92	0.99	0.99	0.95	0.97	0.97
28	1.33, 1.88	2.23, 2.71	1.32, 1.86	1.32, 1.88	1.29, 1.79	1.30, 1.88
28a	3.50, 3.86	1.16, 1.22	3.46, 3.78	3.55, 3.85	3.40, 3.74	3.81
29	4.60, 4.70	4.64, 4.76	4.57, 4.69	4.57, 4.68	4.58, 4.69	4.58, 4.69
30	1.68	1.72	1.68	1.68	1.68	1.69
monosaccharide unit:						
1	5.13	6.40	4.73	4.25	4.72	4.89
2	5.23	5.72	3.77	3.62	3.86	3.66
3	5.62	5.86	3.68	3.60	3.70	3.86
4	5.38	6.17-6.08	3.60	3.89	3.39	3.80
5	4.88	4.53-4.43	3.52	3.54, 3.95	3.63	4.06
6	4.57, 4.70	4.70, 4.53-4.43	3.70, 3.82	–	1.32	3.89

[a]Recorded in $CDCl_3$ at 600 MHz, δ in ppm. [b]3-*O*-Acetyl group: OCH_3 2.04 (s) ppm. [c]Recorded in $CDCl_3$ / CD_3OD mixture (1:1) at 600 MHz, δ in ppm.

Table 2. ^{13}C NMR spectroscopic data for compounds **7**-**10**, **20**-**23,** and **25**-**29**.

Atom No.	**7** [a,b]	**8** [a]	**9** [a,b]	**10** [a]	**20** [a,b,c]	**21** [a,b,c]	**22** [a,b,c]
triterpene unit:							
1	38.3	38.7	38.3	38.7	38.4	38.3	38.4
2	23.6	27.3	23.6	27.3	23.7	23.6	23.7
3	80.9	78.98	80.9	79.0	80.9	80.9	80.9
4	37.7	38.8	37.7	38.8	37.7	37.7	37.8
5	55.3	55.3	55.3	55.3	55.4	55.3	55.4
6	18.1	18.3	18.1	18.2	18.2	18.1	18.2
7	34.1	34.2	34.0	34.1	34.1	33.9	34.1
8	40.8	40.8	40.7	40.8	40.9	40.7	40.9
9	50.3	50.4	50.2	50.3	50.4	50.2	50.4
10	37.0	37.1	37.0	37.1	37.0	37.0	37.1
11	20.9	20.9	20.8	20.8	20.9	20.8	21.0
12	25.0	25.1	24.9	25.1	25.1	24.9	25.0
13	37.1	37.12	37.4	37.5	37.2	37.0	37.2
14	42.5	42.5	42.5	42.6	42.5	42.3	42.5
15	27.3	27.3	27.2	27.2	27.3	27.1	27.3
16	31.5	31.5	31.5	31.5	31.4	31.3	27.0
17	44.7	44.7	46.1	46.1	44.8	44.6	44.7
18	50.1	50.1	49.8	49.9	50.1	49.9	50.1
19	47.3	47.3	47.3	47.3	47.3	47.2	47.3
20	150.6	150.6	150.1	150.5	150.5	150.5	150.5
21	29.9	29.9	29.6	29.7	29.9	29.8	29.9
22	36.1	36.1	36.2	36.2	36.1	35.9	35.8
23	27.9	27.9	27.9	27.9	27.9	27.9	27.9
24	16.4	15.3	16.4	15.3	16.4	16.4	16.4
25	16.1	16.1	16.1	16.1	16.1	16.1	16.2
26	16.0	16.1	15.9	16.0	16.2	16.0	16.2
27	14.8	14.8	14.8	14.9	14.8	14.7	14.9
28	30.5	30.5	33.4	33.4	26.9	27.0	27.1
28a	60.0	60.1	178.9	178.7	65.9	67.4	65.5
29	109.5	109.5	109.8	109.7	109.7	109.5	109.6
30	19.2	19.3	19.3	19.3	19.3	19.4	19.3
monosaccharide unit:							
1	–	–	–	–	97.6	101.1	97.5
2	–	–	–	–	70.6	69.9	71.0
3	–	–	–	–	70.0	70.7	70.0
4	–	–	–	–	67.1	68.5	71.9
5	–	–	–	–	68.9	62.7	66.8
6	–	–	–	–	63.1	–	17.7

[a]Recorded in $CDCl_3$ at 150 MHz, δ in ppm. [b]3-*O*-Acetyl group: *C*OCH_3 = 171.0 ppm, CO*C*H_3 = 21.3 ppm. [c] C=O groups: 165-166 ppm, aromatic carbons: 128-133 ppm. [d]Recorded in $CDCl_3$ / CD_3OD mixture (1:1) at 150 MHz, δ in ppm.

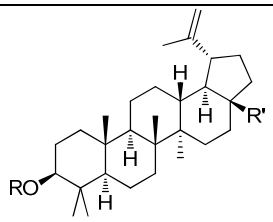
Table 2 contd. ^{13}C NMR spectroscopic data for compounds **7**-**10**, **20**-**23,** and **25**-**29**.

Atom No.	**23** [a,b,c]	**25** [a,b,c]	**26** [d]	**27**[d]	**28** [d]	**29** [d]
triterpene unit:						
1	38.3	38.3	40.0	38.6	38.5	38.4
2	23.7	23.7	28.0	26.9	26.8	26.5
3	80.9	80.9	79.6	78.7	78.6	78.3
4	37.8	37.8	39.4	38.7	38.6	38.5
5	55.3	55.4	56.8	55.6	55.1	55.0
6	18.1	18.2	19.4	18.1	18.1	17.9
7	34.1	34.1	35.4	34.1	34.0	33.9
8	40.8	40.8	42.1	40.7	40.6	40.5
9	50.3	50.2	51.8	50.3	50.2	50.1
10	37.0	37.0	38.3	37.0	36.9	36.7
11	20.9	20.9	22.1	20.8	20.7	20.5
12	25.0	25.0	26.4	24.9	24.9	24.7
13	37.1	37.6	38.6	37.1	37.0	36.8
14	42.4	42.6	43.6	42.4	42.3	42.1
15	27.2	27.2	28.5	27.2	27.1	26.9
16	31.3	31.6	32.6	31.3	31.3	31.1
17	44.6	46.6	46.1	44.5	44.5	44.4
18	49.9	49.8	51.3	49.9	49.8	49.7
19	47.4	47.3	48.8	47.2	47.2	47.0
20	150.1	149.8	151.8	150.5	150.4	151.1
21	30.0	29.7	31.1	29.8	29.7	29.5
22	36.1	36.3	37.2	35.9	35.8	35.6
23	27.9	27.9	28.6	27.8	27.6	27.4
24	16.4	16.4	16.1	15.2	15.1	14.9
25	16.1	16.2	16.7	15.97	15.85	15.66
26	16.0	16.1	16.6	16.0	15.81	15.64
27	14.8	14.9	15.4	14.7	14.6	14.4
28	27.1	33.7	28.04	26.7	26.7	26.5
28a	65.5	170.2	65.8	66.7	64.6	65.3
29	109.2	110.1	110.2	109.4	109.3	109.2
30	19.2	19.2	19.6	19.1	19.0	18.8
monosaccharide unit:						
1	100.7	90.5	101.6	102.6	99.7	102.2
2	67.2	69.2	72.3	71.1	70.6	67.9
3	66.94	69.8	72.7	72.6	71.3	68.8
4	66.91	66.3	68.6	67.5	72.7	69.6
5	64.5	71.3	74.7	65.0	67.9	66.5
6	63.7	62.7	62.9	–	17.2	62.1

[a]Recorded in $CDCl_3$ at 150 MHz, δ in ppm. [b]3-*O*-Acetyl group: *C*OCH_3 = 171.0 ppm, CO*C*H_3 = 21.3 ppm. [c] C=O groups: 165-166 ppm, aromatic carbons: 128-133 ppm. [d]Recorded in $CDCl_3$ / CD_3OD mixture (1:1) at 150 MHz, δ in ppm.

Ichopanol (**8**, entry 1) was highly selective against CEM cell lines (IC_{50} 15.3 μM), whereas the parent betulin (**1**, entry 2) was less active. 28a-Homolupeol (**31**, entry 3) was less active than the above compounds, less selective and slightly toxic against normal cells. Such data may suggest that both hydroxyl groups (at C-3 and C-28 / C-28a) are involved in the interactions between triterpene and cell receptors. This speculation may be confirmed by testing of the acetylated derivatives (entries 4-6). 3-*O*-Acetyl-ichopanol (**7**) was less active and less selective than the parent diol **8**, as well as 3-*O*-acetyl-betulin (**32**). Acetylation of both hydroxyls (to afford diacetate **24**) cancelled the activity. All acetates were nontoxic against normal cells.

Table 3. IC_{50} (μM) values obtained from Calcein AM assays with the tested cancer and normal cell lines; means ±SD obtained from three independent experiments performed in triplicate. Betulinic acid (**2**) was used as a positive control.



Entry	R	R'	No	IC50[μM] CEM	MCF7	HeLa	BJ
1	H	–CH_2CH_2OH	**8**	15.3±1.4	>50	>50	>50
2	H	–CH_2OH	**1**[26]	21.2±3.4	>50	>50	48.6±0.1
3	H	–C_2H_5	**31**[27]	30.8±0.6	46.0±5.7	28.5±5.0	45.0±1.3
4	Ac	–CH_2CH_2OH	**7**	19.8±1.9	38.4±0.7	27.9±5.6	>50
5	Ac	–CH_2OH	**32**[26]	30.4±2.2	>50	>50	>50
6	Ac	–CH_2CH_2OAc	**24**	>50	>50	>50	>50
7	H	–$CH(OH)CH_3$	**12**	33.7±5.4	44.9±6.4	37.5±0.6	42.1±0.3
8	Ac	–$CH(OH)CH_3$	**33**[27]	>50	>50	>50	>50
9	Ac	–CH_2CHO	**6**	>50	>50	>50	>50
10	Ac	–CHO	**4**[27]	38.3±15.6	>50	12.3±0.8	>50
11	H	–CHO	**14**	5.4±1.1	37.4±7.2	7.1±0.6	12.6±3.0
12	H	–CH_2CO_2H	**10**	19.4±0.7	>50	42.9±5.5	>50
13	H	–CO_2H	**2**[26]	40.0±2.8	>50	47.6±1.9	>50
14	Ac	–CH_2CO_2H	**9**	9.6±0.6	31.0±1.2	13.5±0.5	24.7±0.1
15	Ac	–CO_2H	**34**[26]	10.0±0.2	21.8±5.5	14.5±4.3	>50
16	Ac	–CH_2CO_2Me	**30**	>50	>50	>50	>50
17	H	–CH_2CH_2O-α-D-Man*p*	**26**	>50	>50	>50	>50
18	H	–CH_2CH_2O-α-D-Ara*p*	**27**	>50	>50	>50	>50
19	H	–CH_2CH_2O-α-L-Rha*p*	**28**	34.0±3.6	>50	23.6±0.1	43.9±2.4
20	H	–CH_2CH_2O-α-D-Ido*p*	**29**	>50	>50	>50	>50

Homobetulin with a secondary hydroxyl at the C-28 position (**12**, entry 7) was less active and less selective than isomeric ichopanol (**8**). Acetylation of the O-3 hydroxyl (**33**, entry 8) removed the activity. It is likely that sterically hindered hydroxyl group at the C-28 position is unable to bind to receptors, and the only possible interactions are between the C-3 hydroxyl group and receptors.

Homologated aldehyde **6** (entry 9) was inactive, whereas betulinal (**14**) was the most active against both CEM and HeLa cell lines (IC_{50} 5.4 and 7.1 μM, respectively), although it was also most toxic to normal cells. Ichopanic acid (**10**) was more active than the parent betulinic acid (**2**, entries 12 and 13, respectively). In these cases, acetylation at the O-3 position increased activity (**9** and **34**, respectively; entries 14 and 15) in comparison to the parent acids. Esterification of the carboxyl group in **9** leading to ester **30** (entry 16) completely suppressed the activity. Cytotoxicity to normal human BJ fibroblasts was usually very low in this group.

Surprisingly, saponins based on the ichopanol core (**26**-**29**) were inactive except L-rhamnoside **28**, which was moderately cytotoxic (IC_{50} 23.6-34.0 μM) and slightly toxic to normal cells (IC_{50} 43.9 μM). Thus, adding a sugar moiety to the O-28a position of homobetulin decreased cytotoxic activity. No such effect has been observed in previously studied betulin derivatives.[26]

These results clearly demonstrate that the C-28 or C-28a primary hydroxyl group is important for the cytotoxicity of (homo)lupane triterpenes. The free hydroxyl at the C-3 position significantly increases cytotoxicity. Unexpectedly, coupling of homolupanes with a sugar moiety at these positions can cause a loss of activity. 28a-Homo-28a-hydroxylupane triterpenes may be considered as interesting starting materials for the preparation of highly cytotoxic derivatives. In comparison to the parent triterpenes they are more cytotoxic and usually nontoxic against normal cells.

3. Conclusion

In conclusion, a series of 28a-homo-28a-hydroxylupane triterpenes and saponins bearing D-mannose, D-idose, L-arabinose, and L-rhamnose moieties were synthesized and evaluated for their cytotoxic activities towards normal and cancer cell lines. Comparison with a series of known lupane triterpenes and saponins revealed that oxygen substituents at the C-28a (or C-28) position affect the cytotoxic properties of these lupanes. Several triterpenes, as well as selected saponins, showed interesting cytotoxic activity profiles against human cancer cell lines.

4. Experimental section

4.1. General

Silica gel HF_{254} and Silica gel 230–400 mesh (E. Merck) were used for TLC and column chromatography, respectively. ^{1}H and ^{13}C NMR spectra were recorded at 298 K with a Varian NMR-vnmrs600 or vnmrs500 spectrometer, using standard experimental conditions and Varian software (ChemPack 4.1). Configurational assignments were based on the NMR measurements, generated using two-dimensional techniques like COSY and ^{1}H-^{13}C gradient selected HSQC (*g*-HSQC), as well as ^{1}H-^{13}C gradient selected HMBC (*g*-HMBC) in several cases. Internal TMS was used as the ^{1}H and ^{13}C NMR chemical shift standard. *J* values are given in Hertz. High-resolution mass spectra (HRMS ESI) were acquired with MARINER and MaldiSYNAPT G2-S HDMS (Waters) mass spectrometers. Optical rotations were measured with a JASCO P-2000 automatic polarimeter. IR spectra were recorded on Jasco 6200 FT-IR spectrophotometer. Vario EL III (Elementar) was used for determination of C and H contents.

Single crystal X-ray diffraction measurements were acquired using an Agilent Supernova diffractometer, at 100 K with graphite monochromated Cu Ka radiation (1.54184 Å). The data were reduced using CrysAlisPRO software[36] then structures were solved by direct methods and refined on F^2 by full-matrix least-squares using SHELXS97 and SHELXL97.[37] All non-hydrogen atoms were refined as anisotropic while hydrogen atoms were placed in calculated positions, and refined in riding mode. Structures contained the solvent (methanol) in 1:1stoichiometry. Both CH_3 (C-30) and CH_2 (C-29) groups connected to the C-20 carbon atom are disordered, with 0.54 and 0.46 occupancy, respectively. *Data for* **8**: orthorombic, $P22_12_1$, a=6.9273(3) b=12.2412(4) c=33.8983(13) Å, V=2874.52(19) $Å^3$, Z=4, D_{calc}=1.129g cm^{-1}, m=0.532 mm^{-1}, R1=0.04320 for 4260Fo, [Fo > 4s(Fo)] and 0.0493 for all data, wR2=0.1092, S=1.038. Crystallographic data (excluding structure factors) for the structure described in this paper have been deposited with the Cambridge Crystallographic Data Centre as supplementary material (deposition number: CCDC 996102).

4.2. Synthesis

4.2.1. *3β*-O-*Acetyl-17-(2-methoxyethenyl)-28-norlup-20(29)-en-3-ol (**5**).* To an ice cooled suspension of (methoxymethyl)triphenylphosphonium chloride (1.51 g, 4.40 mmol) in anhydrous THF (15 mL), *n*-butyllithium (1.6 M in hexane, 1.41 mL, 3.52 mmol) was added dropwise and stirred for 30 min. Then, a solution of 3-*O*-acetylbetulinal (**4**, 429 mg, 0.88 mmol) in anhydrous THF (5 mL) was added, the whole mixture was stirred at 5 °C for an additional 15 min, and for 1 h at r.t. The reaction was quenched with a saturated solution of NH_4Cl (0.5 mL), a small portion of silica gel was added and the solvents were evaporated under diminished pressure. Column chromatography of the residue (hexane – ethyl acetate, 40:1 → 10:1) yielded *the title compound* as a mixture of *(E)*- and *(Z)*-isomers (white solid, 550 mg, 66%). An analytical sample of pure *(E)*-isomer was isolated and had the following physicochemical properties: $[\alpha]_D^{20}$ 17.2 (*c* 0.2, $CHCl_3$); ν_{max} (film): 2952, 2871, 1731, 1715, 1451, 1375, 1247, 1024, 980, 896, 757 cm^{-1}. ^1H NMR (500 MHz, $CDCl_3$) δ: 5.78 (d, 1 H, *J* 6.9 Hz, -CH=C*H*OCH_3), 4.68 (br s, 1 H, H-29), 4.56 (br s, 1 H, H-29), 4.46 (dd, 1 H, *J* 10.8, 5.5 Hz, H-3), 4.28 (d, 1 H, *J* 7.1 Hz, -C*H*=$CHOCH_3$), 3.55 (s, 3 H, -CH=CHOC*H*$_3$), 2.37-2.39 (m, 1 H, H-19), 2.20-2.22 (m, 1 H), 2.04 (s, 3 H, CH_3), 1.68 (s, 3 H, CH_3), 1.61-1.64 (m, 5 H), 1.28-1.48 (m, 8 H), 1.07-1.20 (m, 10 H), 0.97 (s, 3 H, CH_3), 0.93 (s, 3 H, CH_3), 0.85 (s, 3 H, CH_3), 0.84 (s, 3 H, CH_3), 0.83 (s, 3 H, CH_3); ^{13}C NMR (500 MHz, $CDCl_3$) δ: 170.9 (O*C*OCH_3), 151.0 (C-20), 149.8, 145.8, 109.6 (C-29), 104.0, 80.8 (C-3), 59.5, 55.4, 50.3, 48.1, 45.6, 44.5, 42.5, 41.7 (CH_2), 40.7, 38.4, 37.9 (CH_2), 37.1, 36.5, 35.8 (CH_2), 33.6 (CH_2), 30.0 (CH_2), 29.1, 27.4, 26.4 (CH_2), 24.4 (CH_2), 23.2 (CH_2), 20.3 (CH_2), 17.6 (CH_2), 16.0, 15.7, 15.5, 14.4. HR MS-EI calcd. for $C_{34}H_{54}O_3$: 510.4073. Found 510.4067.

4.2.2. *3β*-O-*Acetyl-28a-homobetulinal (**6**).* PPTS (4.0 g) was added to **a** solution of **5** (3.00 g, 5.87 mmol) in acetone (15 mL) and water (1.5 mL). The solution was heated at 60 °C for 48 h, then the solvents were evaporated. Column chromatography (hexane – ethyl acetate, 40:1 → 20:1) of the residue gave **6** as a white foam (1.99 g, 68%). $[\alpha]_D^{20}$ 13.7 (*c* 0.2, $CHCl_3$). ν_{max} (film): 2947, 2871, 1723, 1453, 1374, 1247, 1028, 980, 886, 757 cm^{-1}. ^1H NMR (500 MHz, $CDCl_3$) δ: 9.83-9.85 (m, 1 H, CHO), 4.69 (1 H, H-29), 4.60 (br s, 1 H, H-29), 4.47 (dd, 1 H, *J* 10.7, 5.5 Hz, H-3), 2.53-2.56 (m, 1 H, H-28), 2.33-2.36 (m, 1 H, H-19), 2.09-2.10 (m, 1 H), 2.04 (s, 3 H, CH_3), 1.83-1.85 (m, 2 H), 1.69 (s, 3 H, CH_3), 1.60-1.66 (m, 6 H), 1.39-1.50 (m, 9 H), 1.24-1.29 (m, 3 H), 1.09-1.10 (m, 2 H), 1.04 (s, 3 H, CH_3), 0.97-0.98 (m, 2 H), 0.97 (s, 3 H, CH_3), 0.85 (s, 3 H, CH_3), 0.84 (s, 3 H, CH_3), 0.83 (s, 3 H, CH_3). ^{13}C NMR (500 MHz, $CDCl_3$) δ: 204.1 (CHO), 171.0 (C=O), 149.8 (C-20), 110.1 (C-29), 80.8 (C-3), 55.3, 50.2, 50.1, 47.5, 45.6 (C), 42.4 (C), 42.2 (CH_2), 40.8 (C), 38.4 (CH_2), 37.7 (C), 37.5, 37.05 (C), 36.3 (CH_2), 34.1 (CH_2), 32.1 (CH_2), 29.5 (CH_2), 27.9, 26.9 (CH_2), 24.9 (CH_2), 23.6 (CH_2), 21.2, 20.8 (CH_2), 19.3 (C), 18.1 (CH_2), 16.4, 16.1, 16.0, 14.8. HR MS-EI calcd. for $C_{33}H_{52}O_3$: 496.3916. Found 496.3912.

4.2.3. *3β-O-Acetyl-28a-homobetulin (**7**)*. To a solution of **6** (950 mg, 1.91 mmol) in MeOH (10 mL), $NaBH_4$ (217 mg, 5.74 mmol) was added. The solution was stirred at r.t. for 30 min. and concentrated to dryness. Column chromatography (hexane – ethyl acetate, 20:1 → 5:1) of the residue gave **7** (803 mg, 84%) as a white solid; m.p. 191-192 °C; $[\alpha]_D^{20}$ 20.4 (*c* 0.2, $CHCl_3$). ν_{max} (film): 3418, 2946, 2871, 1732, 1454, 1375, 1247, 1027, 980, 882, 739 cm^{-1}. ^1H NMR data, see Table 1; ^{13}C NMR data, see Table 2. Anal. Calcd. for $C_{33}H_{54}O_3$ (498.78): C, 79.46; H, 10.91. Found: C 79.42; H 10.76.

4.2.4. *28a-Homobetulin (**8**)*. To a solution of **7** (80 mg, 0.16 mmol) in EtOH (2 mL), potassium hydroxide (23 mg, 0.40 mmol) was added, and the mixture was refluxed for 1 h, then the reaction mixture was cooled to room temperature, the solvent was evaporated to dryness, and the residue was purified by column chromatography (hexane – ethyl acetate, 20:1 → 1:1) to give **8** as a white solid (73 mg, quant.); m.p. 204-206 °C; $[\alpha]_D^{20}$ 15.9 (*c* 0.2, $CHCl_3$). ν_{max} (film): 3342, 2942, 2869, 1454, 1376, 1045, 883, 758 cm^{-1}. ^1H NMR data, see Table 1; ^{13}C NMR data, see Table 2. Anal. Calcd. for $C_{31}H_{52}O_2 \times CH_3OH$ (488.78): C, 78.63; H, 11.55. Found: C, 78.87; H, 11.48; HR MS-EI calcd. for $C_{31}H_{52}O_2$: 456.3980. Found 456.3967.

4.2.5. *3β-O-Acetyl-28a-homobetulinic acid (**9**)*. To an iced cooled solution of **6** (100 mg, 0.2 mmol) in acetone (2 mL) and THF (2 mL) Jones reagent (3 mL) was added, the mixture was stirred at r.t. for 5 h. The reaction was quenched with isopropyl alcohol (1.5 mL) solvents were decanted from the gummy residue and evaporated under diminished pressure. Column chromatography (hexane – ethyl acetate, 20:1 → 7:3) of the residue gave *the title compound* as a foam (87 mg, 84%). $[\alpha]_D^{20}$ 22.7 (*c* 0.3, $CHCl_3$). ν_{max} (film): 2946, 2872, 1732, 1703, 1454, 1376, 1247, 1029, 980, 884, 758 cm^{-1}. ^1H NMR data, see Table 1; ^{13}C NMR data, see Table 2. Anal. Calcd. for $C_{33}H_{52}O_4 \times H_2O$ (530.76): C, 74.67; H, 10.25 Found: C, 74.69; H, 10.19.

4.2.6. *28a-Homobetulinic acid (**10**)*. To a solution of **9** (56 mg, 0.11 mmol) in EtOH (2.5 mL), potassium hydroxide (15 mg, 0.27 mmol) was added, and the mixture was refluxed for 3 h, then cooled to r.t. and the solvents were evaporated to dryness. Column chromatography (hexane – ethyl acetate, 10:1 → 1:1) of the residue gave **10** as a white solid (40 mg, 77%); m.p. 199-202 °C; $[\alpha]_D^{20}$ 12.9 (*c* 0.2, $CHCl_3$). ν_{max} (film): 2943, 2871, 1704, 1453, 1377, 758 cm^{-1}. ^1H NMR data, see Table 1; ^{13}C NMR data, see Table 2. HR MS-ESI calcd. for $C_{31}H_{49}O_3$ $[M-H]^-$: 469.3687. Found 469.3682.

4.2.7. *28-C-Methyl-betulin (**12**)*. To a solution of 3-*O*-allyl-28-*C*-methyl-betulin[27] (**11**, main isomer from the Grignard reaction, 550 mg, 1.11 mmol) in methanol (10 mL) and DCM (5 mL), palladium(II) chloride (50 mg) was added and the mixture was stirred at r.t. overnight. The solvents were evaporated to dryness. Column chromatography of the residue (hexane – ethyl acetate, 10:1 → 1:1) afforded **12** (359 mg, 71%) as white foam. $[\alpha]_D^{20}$ 11.1 (*c* 1.0, $CHCl_3$). ν_{max} (film): 3409, 2941, 2870, 1452, 1375, 1042, 883, 757 cm^{-1}. ^1H NMR (500 MHz, $CDCl_3$) δ: 4.71 (d, 1 H, *J* 2.2 Hz, H-29), 4.57 (dd, 1 H, *J* 2.3, 1.3 Hz, H-29), 4.33 (q, 1 H, *J* 6.3 Hz, H-28), 3.19 (dd, 1 H, *J* 4.9, 11.4 Hz, H-3), 2.84-2.90 (m, 1 H), 2.05-2.10 (m, 1 H), 1.90-1.97 (m, 1

H), 1.82-1.87 (m, 1 H), 1.69 (s, 3 H, CH_3), 1.18 (d, 3 H, *J* 6.3 Hz, H-28a), 1.04 (s, 3 H, CH_3), 1.01 (s, 3 H, CH_3), 0.97 (s, 3 H, CH_3), 0.83 (s, 3 H, CH_3), 0.76 (s, 3 H, CH_3), 0.68-1.73 (m, 22 H, lupane protons). ^{13}C NMR (500 MHz, $CDCl_3$) δ: 151.4 (C-20), 109.4 (C-29), 79.0, 68.3, 55.3, 50.4, 50.2, 50.1 (C), 49.0, 42.9 (C), 40.9 (C), 38.8 (C), 38.7 (CH_2), 37.1 (C), 36.9, 34.6 (CH_2), 34.2 (CH_2), 32.8 (CH_2), 32.2 (CH_2), 28.0, 27.7 (CH_2), 27.4 (CH_2), 25.2 (CH_2), 20.9 (CH_2), 19.8, 18.9 (C), 18.3 (CH_2), 16.1, 16.1, 15.3, 15.2. HR-MS (ESI) calc. for $C_{31}H_{52}O_2$ $(M)^+$: 456.3967. Found: 456.3970. Anal. Calcd for $C_{31}H_{52}O_2 \times H_2O$ (474.77): C, 78.43; H, 11.46. Found: C, 78.42; H, 11.37.

4.2.8. *Betulinal (**14**)*. To a solution of 3-*O*-allyl-betulinal (**13**, 100 mg, 0.20 mmol) in methanol (8 mL) and DCM (4 mL), palladium(II) chloride (20 mg) was added and the mixture was stirred at r.t. overnight. The solvents were evaporated to dryness. Column chromatography of the residue (hexane – ethyl acetate, 20:1) afforded *the title compound* (64 mg, 80%) as white foam. ν_{max} (film): 3409, 2941, 2869, 1691, 1452, 1388, 1370, 1041, 1030, 756 cm^{-1}. NMR spectroscopic data of **14** matches the literature.[38]

4.3. General method for glycosylation

A solution of glycosyl donor (0.24 mmol) and the corresponding triterpene (0.20 mmol) in CH_2Cl_2 (10 mL) was stirred for 20 min. at r.t. over molecular sieves (4 Å, 200 mg, finely ground), then cooled to –40 °C and TMSOTf (20 µL, 0.1 mmol) was added. After 25 min the reaction was quenched with Et_3N (1 mL), and the solvents were evaporated under diminished pressure. Column chromatography (hexane – ethyl acetate, 40:1 → 5:1) of the residue gave the protected saponins as white foams.

4.3.1. *3β-*O*-Acetyl-28a-*O*-(2,3,4,6-tetra-*O*-benzoyl-α-D-mannopyranosyl)-28a-homolup-20(29)-ene (**20**)*. Yield 89% (191 mg). ν_{max} (film): 2947, 2871, 1730, 1451, 1265, 1108, 1096, 1069, 1028, 978, 757, 711 cm^{-1}. $[\alpha]_D^{20}$ –28.2 (*c* 0.3, $CHCl_3$). ^{1}H NMR data, see Table 1; ^{13}C NMR data, see Table 2. Anal. Calcd. for $C_{67}H_{80}O_{12}$ (1077.34): C, 74.69; H, 7.53 Found: C, 74.55; H, 7.53.

4.3.2. *3β-*O*-Acetyl-28a-*O*-(2,3,4-tri-*O*-benzoyl-α-D-arabinopyranosyl)-28a-homolup-20(29)-ene (**21**)*. Yield 92% (173 mg). $[\alpha]_D^{20}$ –62.1 (*c* 0.3, $CHCl_3$). ν_{max} (film): 2946, 2871, 1730, 1452, 1282, 1262, 1251, 1093, 1063, 1028, 757, 711 cm^{-1}. ^{1}H NMR data, see Table 1; ^{13}C NMR data, see Table 2. Anal. Calcd. for $C_{59}H_{74}O_{10}$ (943.21): C, 75.13; H, 7.91 Found: C, 75.21; H, 8.08.

4.3.3. *3β-*O*-Acetyl-28a-*O*-(2,3,4-tri-*O*-benzoyl-α-L-rhamnopyranosyl)-28a-homolup-20(29)-ene (**22**)*. Yield 99% (190 mg). $[\alpha]_D^{20}$ 71.4 (*c* 0.3, $CHCl_3$). ν_{max} (film): 2946, 2872, 1731, 1451, 1278, 1263, 1248, 1108, 1069, 1028, 979, 757, 711 cm^{-1}. ^{1}H NMR data, see Table 1; ^{13}C NMR data, see Table 2. HR MS-ESI calcd. for $C_{60}H_{80}NO_{10}$ $[M+NH_4]^+$: 974.5782. Found 974.5782.

4.3.4. *3β*-O-*Acetyl-28a*-O-*(2,3,4,6-tetra*-O-*benzoyl-α-D-idopyranosyl)-28a-homolup-20(29)-ene* ***(23)***. Yield 97% (209 mg). $[\alpha]_D^{20}$ 40.9 (*c* 0.2, $CHCl_3$). ν_{max} (film): 2947, 2871, 1727, 1263, 1248, 1108, 1069, 1027, 756, 710 cm^{-1}. ^1H NMR data, see Table 1; ^{13}C NMR data, see Table 2. Anal. Calcd. for $C_{67}H_{80}O_{12}$ (1077.34): C, 74.69; H, 7.48 Found: C, 74.70; H, 7.68.

4.3.5. *3β,28a-Di*-O-*acetyl-28a-homobetulin* ***(24)***. Yield 83% (90 mg), white solid; m.p. 224-226 °C. $[\alpha]_D^{20}$ 21.0 (*c* 0.2, $CHCl_3$). ν_{max} (film): 2946, 2871, 1737, 1455, 1366, 1246, 1029, 980, 883, 757 cm^{-1}. ^1H NMR (500 MHz, $CDCl_3$) δ: 4.67-4.68 (m, 1 H, H-29), 4.57-4.58 (m, 1 H, H-29), 4.46 (dd, 1 H, *J* 10.8, *J* 5.5 Hz, H-3), 4.12 (ddd, 1 H, *J* 6.1, *J* 10.2, *J* 10.2 Hz, H-28a), 4.02 (ddd, 1 H, *J* 5.5, *J* 10.2, *J* 10.2 Hz, H-28a), 2.37-2.41 (m, 1 H, H-19), 2.05 (s, 3 H, CH_3), 2.04 (s, 3 H, CH_3), 1.85-1.95 (m, 3 H), 1.78-1.73 (m, 2 H), 1.68 (s, 3 H, CH_3), 1.58-1.66 (m, 6 H), 1.35-1.51 (m, 5 H), 1.21-1.31 (m, 5 H), 1.08-1.10 (m, 2 H), 1.04 (s, 3 H, CH_3), 1.01 (m, 2 H), 0.95 (s, 3 H, CH_3), 0.85 (s, 3 H, CH_3), 0.84 (s, 3 H, CH_3), 0.83 (s, 3 H, CH_3), 0.79 (m, 1 H). ^{13}C NMR (500 MHz, $CDCl_3$) δ: 171.2 (C=O), 170.9 (C=O), 150.4 (C-20), 109.6 (C-29), 80.9 (C-3), 62.2, 55.3 (CH_2), 50.3 (CH_2), 50.0 (CH_2), 47.3 (CH_2), 44.7 (C), 42.5 (C), 40.8 (C), 38.3, 37.7 (C), 37.1 (CH_2), 37.0 (C), 35.9, 34.1, 31.3, 29.8, 27.9 (CH_2), 27.2, 26.0, 25.0, 23.6, 21.3 (CH_2), 21.1 (CH_2), 20.9, 19.2, 18.1, 16.4 (CH_2), 16.1 (CH_2), 16.0 (CH_2), 14.8. Anal. Calcd. for $C_{35}H_{56}O_4$ (540.82): C, 77.73; H, 10.44. Found: C, 77.69; H, 10.30.

4.3.6. *1*-O-*[3β*-O-*Acetyl-28a-homolup-20(29)-ene-28a-oyl)-2,3,4,6-tetra*-O-*benzoyl-α-D-mannopyranosyl* ***(25)***. Yield 97% (212 mg). $[\alpha]_D^{20}$ –20.9 (*c* 0.3, $CHCl_3$). ν_{max} (film): 2947, 2871, 1731, 1452, 1263, 1107, 1095, 1027, 978, 757, 710 cm^{-1}. ^1H NMR data, see Table 1; ^{13}C NMR data, see Table 2. Anal. Calcd. for $C_{67}H_{78}O_{13}$ (1091.33): C, 73.74; H, 7.20. Found: C, 73.61; H, 7.39.

4.5. General procedure for the debenzoylation reaction

A suspension of protected saponin (0.10 mmol) and K_2CO_3 (20 mg) in MeOH (3 mL) was stirred for 48 h, then neutralized with Amberlyst 15 resin (H^+ form) and filtered through a PTFE syringe filter (MeOH as eluent), and the filtrate was evaporated to dryness. The residue was purified by column chromatography to afford unprotected saponin as white foam.

4.5.1. *28a*-O-*(α-D-Mannopyranosyl)-28a-homobetulin* ***(26)***. Column chromatography (hexane – ethyl acetate, 10:1 → 1:1, and hexane – ethyl acetate – methanol, 5:3:1). ν_{max} (film): 3348, 2941, 2870, 1563, 1454, 1380, 1132, 1082, 1046, 1032, 981, 880, 681 cm^{-1}. Yield 94% (78 mg). $[\alpha]_D^{20}$ 29.8 (*c* 0.2, $CHCl_3$-CH_3OH). ^1H NMR data, see Table 1; ^{13}C NMR data, see Table 2. HR MS-ESI calcd. for $C_{37}H_{62}NaO_7$ $[M+Na]^+$: 641.4392. Found 641.4393.

4.5.2. *28a*-O-*(α-D-Arabinopyranosyl)-28a-homobetulin* ***(27)***. Column chromatography (hexane – ethyl acetate, 7:3 → 1:1, and hexane – ethyl acetate – methanol, 5:3:1). Yield 78% (66 mg). $[\alpha]_D^{20}$ 6.2 (*c* 0.3,

$CHCl_3$-CH_3OH). ν_{max} (film): 3387, 2942, 2869, 1451, 1375, 1070, 1040, 996, 878, 755 cm^{-1}. ^1H NMR data, see Table 1; ^{13}C NMR data, see Table 2. HR MS-ESI calcd. for $C_{36}H_{60}NaO_6$ $[M+Na]^+$: 611.4287. Found 611.4288.

4.5.3. *28a*-O-*(α-L-Rhamnopyranosyl)-28a-homobetulin (**28**).* Column chromatography (hexane – ethyl acetate, 40:1 → 5:1, and hexane – ethyl acetate – methanol, 5:3:1). Yield 56% (55 mg). $[\alpha]_D^{20}$ –20.5 (*c* 0.3, $CHCl_3$-CH_3OH). ν_{max} (film): 3374, 2943, 2870, 1466, 1454, 1389, 1376, 1134, 1062, 1044, 983, 880 cm^{-1}. ^1H NMR data, see Table 1; ^{13}C NMR data, see Table 2. HR MS-ESI calcd. for $C_{37}H_{62}NaO_6$ $[M+Na]^+$: 625.4442. Found 625.4444.

4.5.4. *28a*-O-*(α-D-Idopyranosyl)-28a-homobetulin (**29**).* Column chromatography (hexane – ethyl acetate, 10:1 → 1:1, and hexane – ethyl acetate – methanol, 5:3:1). Yield 86% (84 mg). $[\alpha]_D^{20}$ 32.2 (*c* 0.3, $CHCl_3$-CH_3OH). ν_{max} (film): 3388, 2941, 2869, 1453, 1389, 1377, 1093, 1026, 879, 718 cm^{-1}. ^1H NMR data, see Table 1; ^{13}C NMR data, see Table 2. HR MS-ESI calcd. for $C_{37}H_{62}NaO_7$ $[M+Na]^+$: 641.4393. Found 641.4411.

4.5.5. *3β*-O-*Acetyl-28a-homobetulinic acid methyl ester (**30**).* A suspension of **25** (80 mg, 0.07 mmol) and K_2CO_3 (40 mg) in MeOH (5 mL) was stirred at r.t. for 1 h, then the solvents were evaporated under diminished pressure. Column chromatography (hexane – ethyl acetate, 40:1 → 10:1) of the residue gave **30** as a white foam (24 mg, 63%). $[\alpha]_D^{20}$ 25.5 (*c* 0.2, $CHCl_3$). ν_{max} (film): 2947, 2871, 1734, 1640, 1454, 1377, 1245, 1019, 980, 884, 758 cm^{-1}. ^1H NMR (500 MHz, $CDCl_3$) δ: 4.68 (br s, 1 H, H-29), 4.58-4.61 (m, 1 H, H-29), 4.47 (dd, 1 H, *J* 10.8, *J* 5.5 Hz, H-3), 3.65 (s, 3 H, CH_3), 2.49-2.52 (m, 1 H, H-28), 2.32-2.36 (m, 1 H, H-19), 2.04 (s, 3 H, CH_3), 1.80-1.95 (3 H), 1.75-1.78 (m, 1 H), 1.69 (s, 3 H, CH_3), 1.50-1.65 (7 H), 1.40 (5 H), 1.20-1.33 (4 H), 1.10-1.16 (3 H), 1.04 (s, 3 H, CH_3), 1.02 (1 H), 0.96 (s, 3 H, CH_3), 0.85 (s, 3 H, CH_3), 0.84 (s, 3 H, CH_3), 0.83 (s, 3 H, CH_3), 0.79-0.80 (m, 1 H). ^{13}C NMR (500 MHz, $CDCl_3$) δ: 173.6 (C=O), 170.9 (C=O), 150.2 (C-20), 109.7 (C-29), 80.9 (C-3), 55.3 (CH_2), 51.2 (CH_2), 50.2 (CH_2), 49.8 (CH_2), 47.3 (CH_2), 46.1 (C), 42.5 (C), 40.8 (C), 38.3, 37.7 (C), 37.4 (CH_2), 37.0 (C), 36.3, 34.0, 33.5, 31.6, 29.7, 27.9 (CH_2), 27.2, 25.0, 23.6, 21.3 (CH_2), 20.8, 19.3 (C), 18.1, 16.4 (CH_2), 16.1 (CH_2), 15.9 (CH_2), 14.8 (CH_2). Anal. Calcd. for $C_{34}H_{54}O_4$ (526.79): C, 77.52; H, 10.33. Found: C, 77.63; H, 10.33.

4.6. Biological evaluation

4.6.1. Cell culture

Stock solutions (10 mmol/L) of the tested compounds were prepared by dissolving an appropriate quantity of each substance in dimethylsulfoxide (DMSO). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), L-glutamine, penicillin and streptomycin were purchased from Sigma (MO, USA). Calcein AM was obtained from Molecular Probes (Invitrogen Corporation, CA, USA). The screening cell lines (T-lymphoblastic leukemia cell line CEM, breast carcinoma cell line MCF7, cervical carcinoma cell line HeLa,

and human fibroblasts BJ) were obtained from the American Type Culture Collection (Manassas, VA, USA). All cell lines were cultured in DMEM medium (Sigma, MO, USA), supplemented with 10% fetal bovine serum, L-glutamine (2 mmol/L), penicillin (10 000 U) and streptomycin (10 mg/mL). The cell lines were maintained under standard cell culture conditions at 37 ºC and 5% CO_2 in a humid environment. Cells were subcultured two or three times a week using the standard trypsinization procedure.

4.6.2. Calcein AM assay

Suspensions of the tested cell lines (ca. 1.0×10^5 cells/mL) were placed in 96-well microtiter plates and after 24 h of stabilization (time zero), the tested compounds were added (in three 20 µL aliquots) in serially diluted concentrations in dimethylsulfoxide (DMSO). Control cultures were treated with DMSO alone, and the final concentration of DMSO in the incubation mixtures never exceeded 0.6%. The test compounds were typically evaluated at six 3-fold dilutions and the highest final concentration was generally 50 µM. After 72 h incubation, Calcein AM solution (100 µL, Molecular Probes, Invitrogen, CA, USA) was added, and incubation was continued for a further hour. The fluorescence of viable cells was then quantified using a Fluoroskan Ascent instrument (Labsystems, Finland). The percentage of surviving cells in each well was calculated by dividing the intensity of the fluorescence signals from the exposed wells by the intensity of signals from control wells and multiplying by 100. These ratios were then used to construct dose-response curves from which IC_{50} values, the concentrations of the respective compounds that were lethal to 50% of the tumor cells, were calculated.

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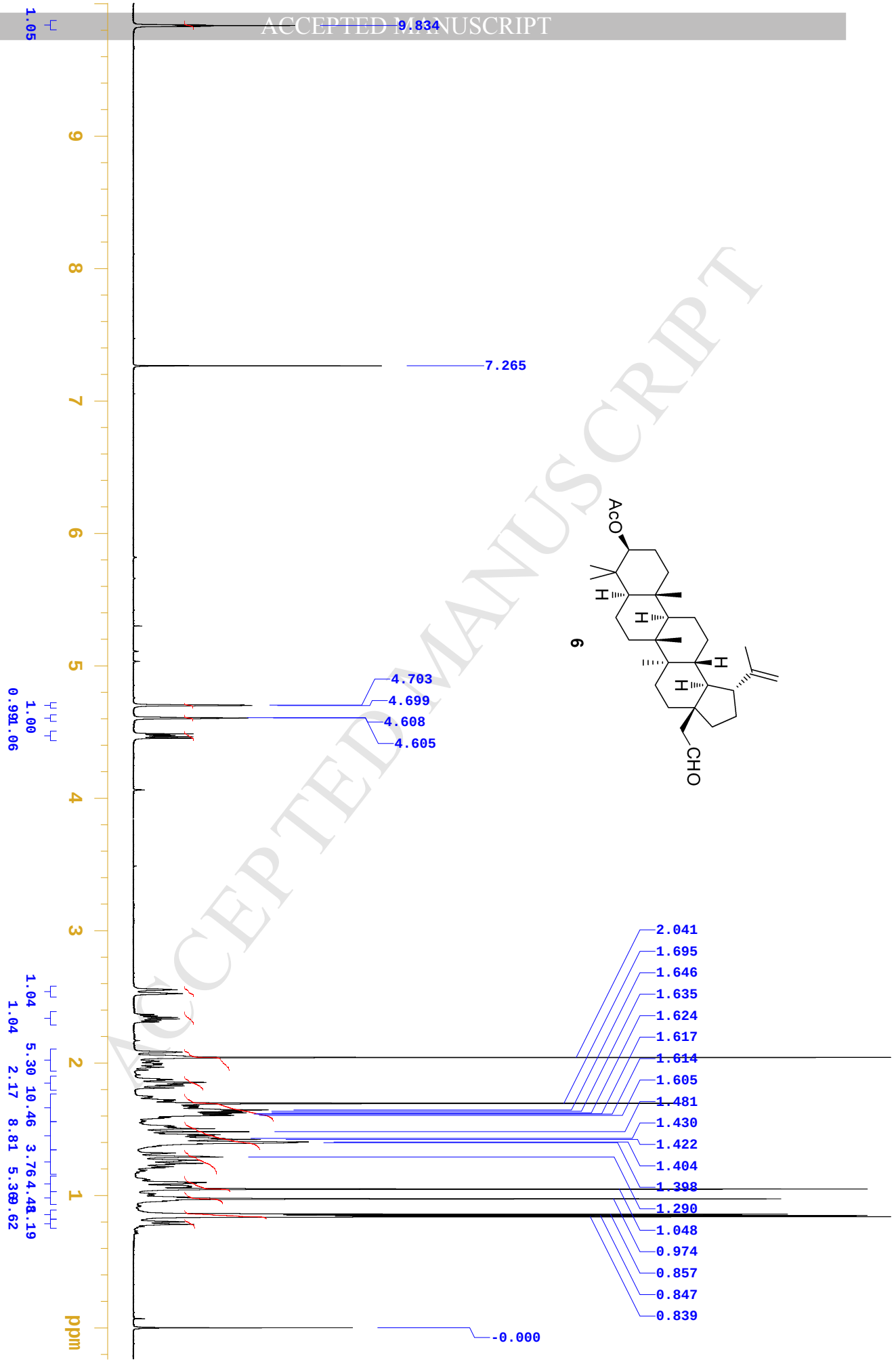

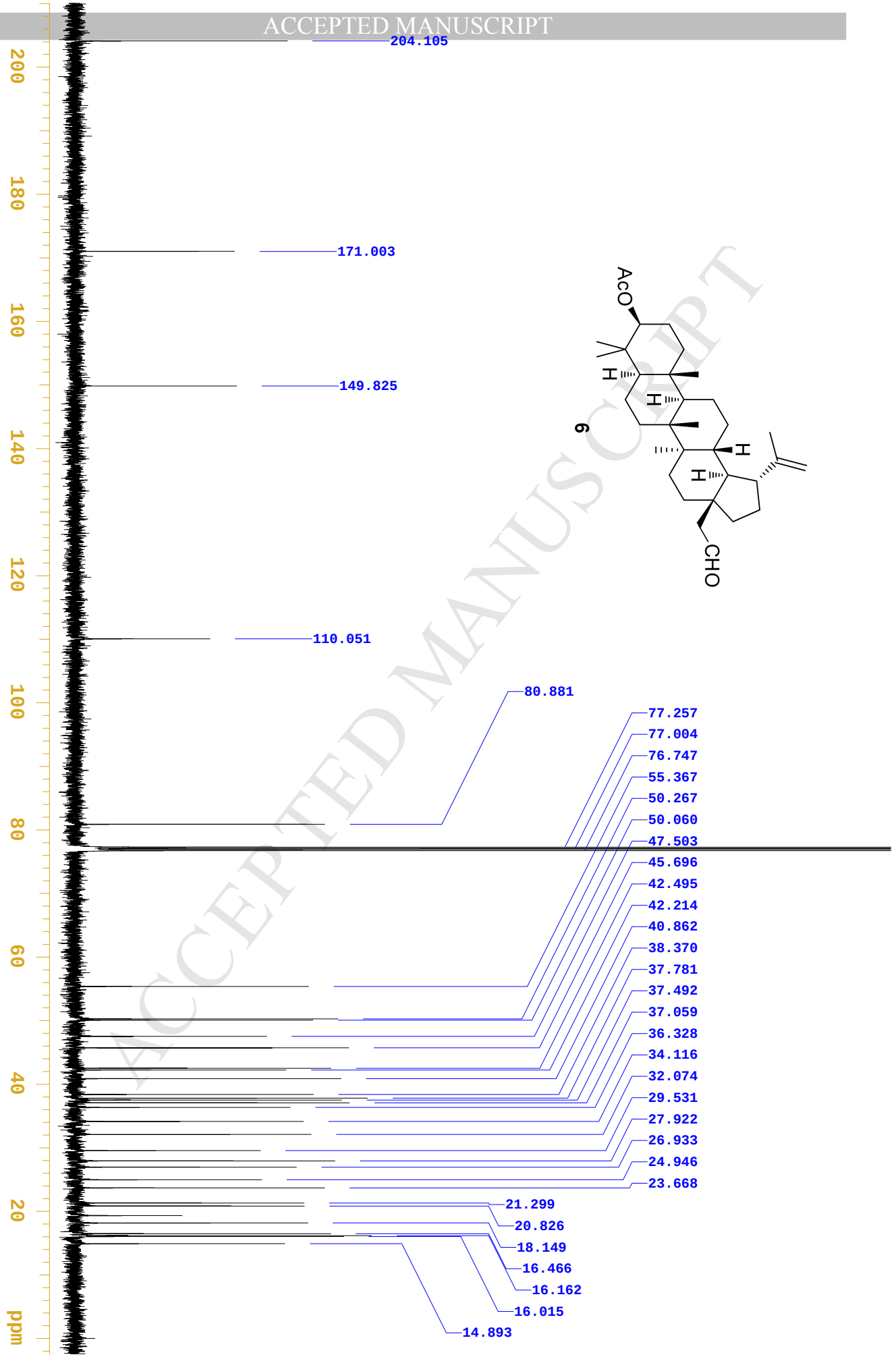

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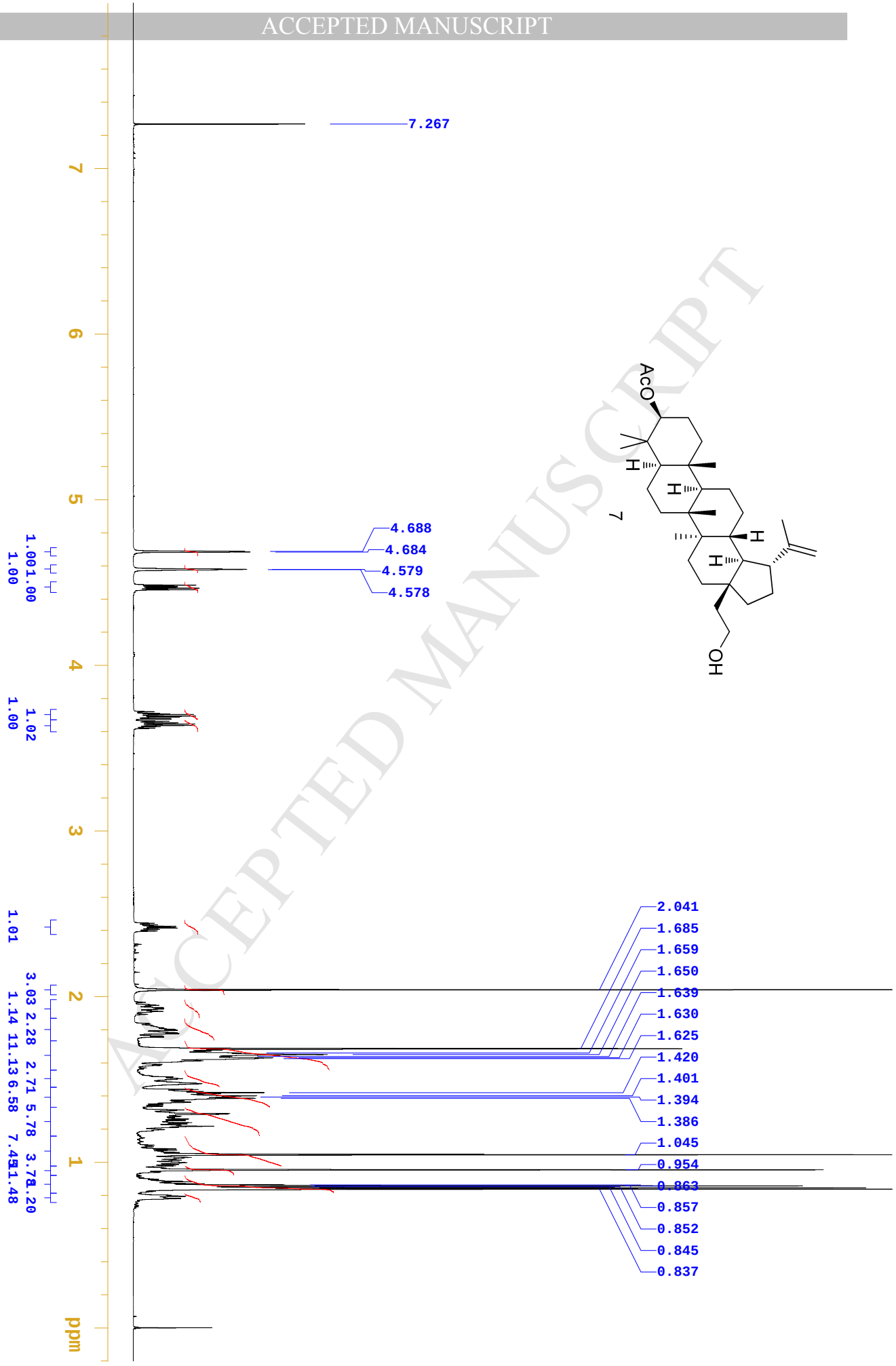

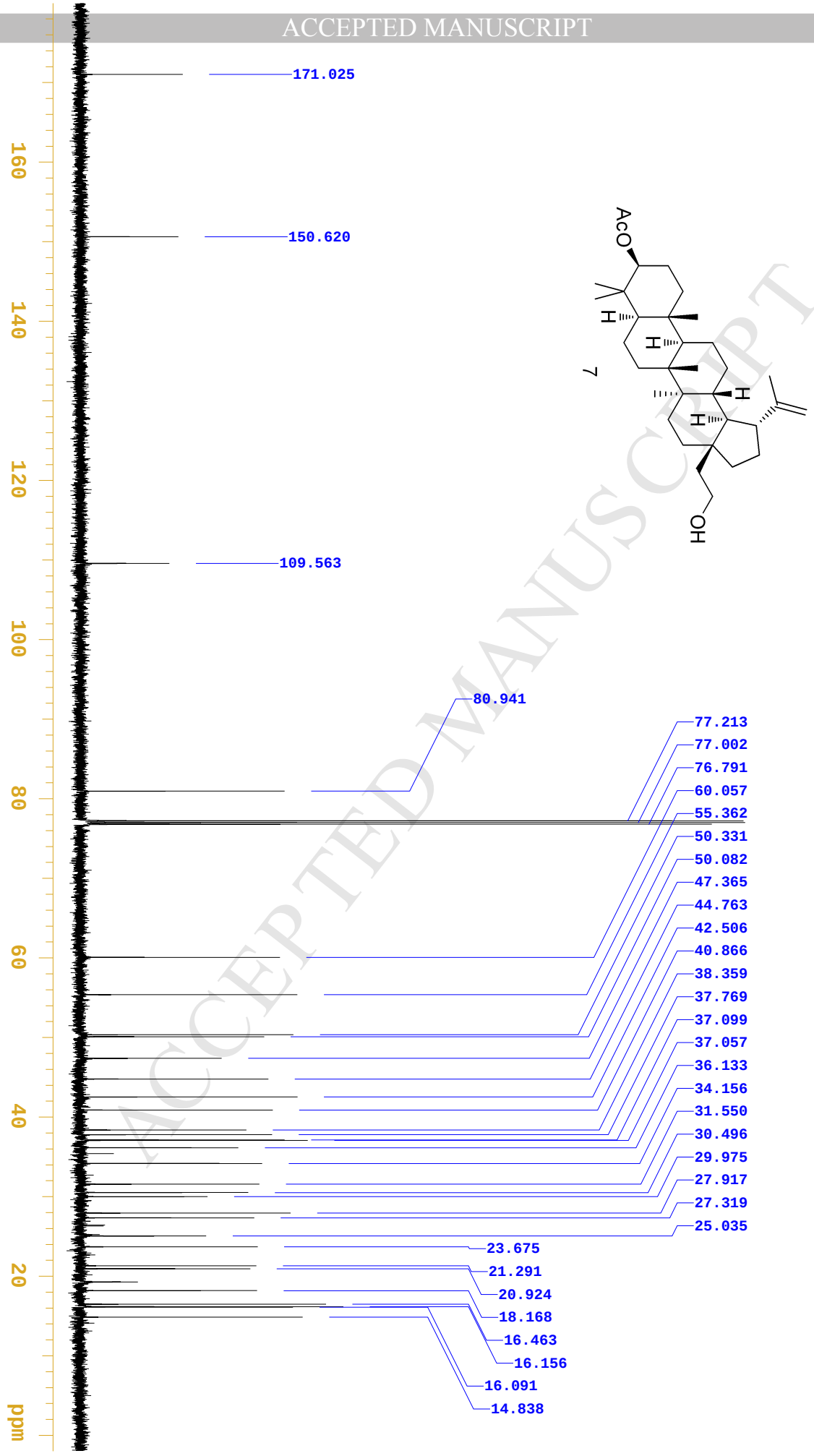

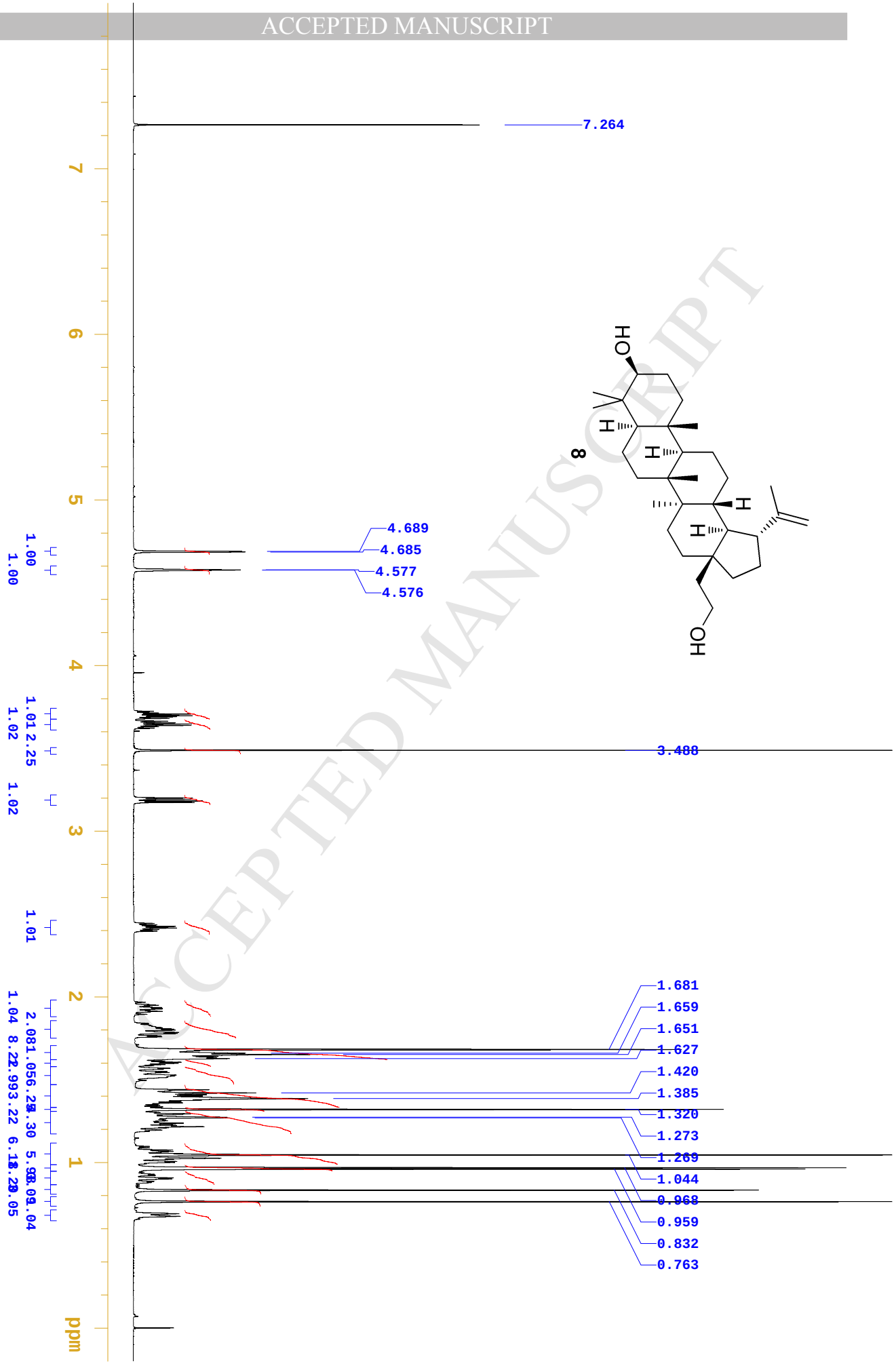

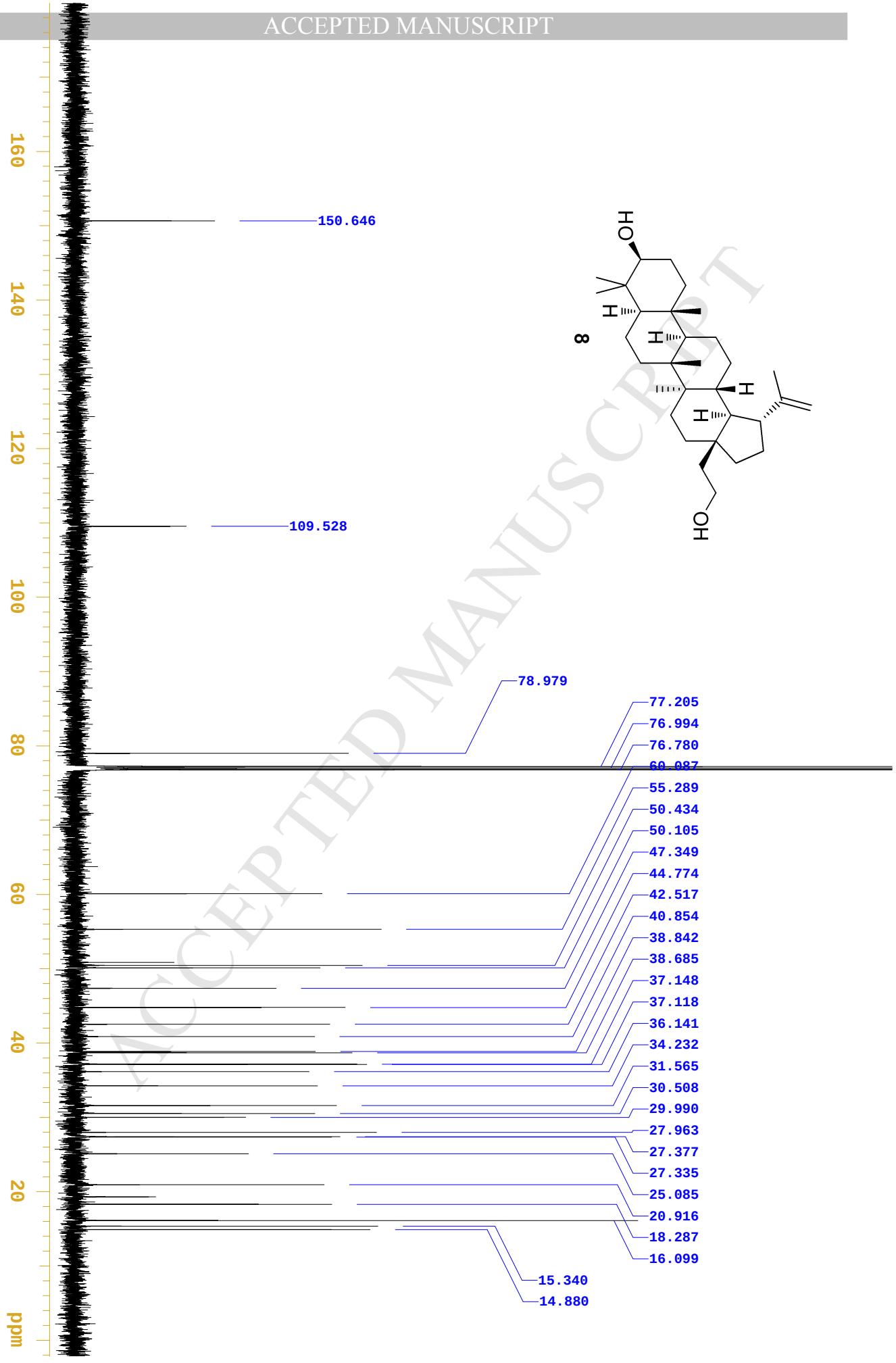

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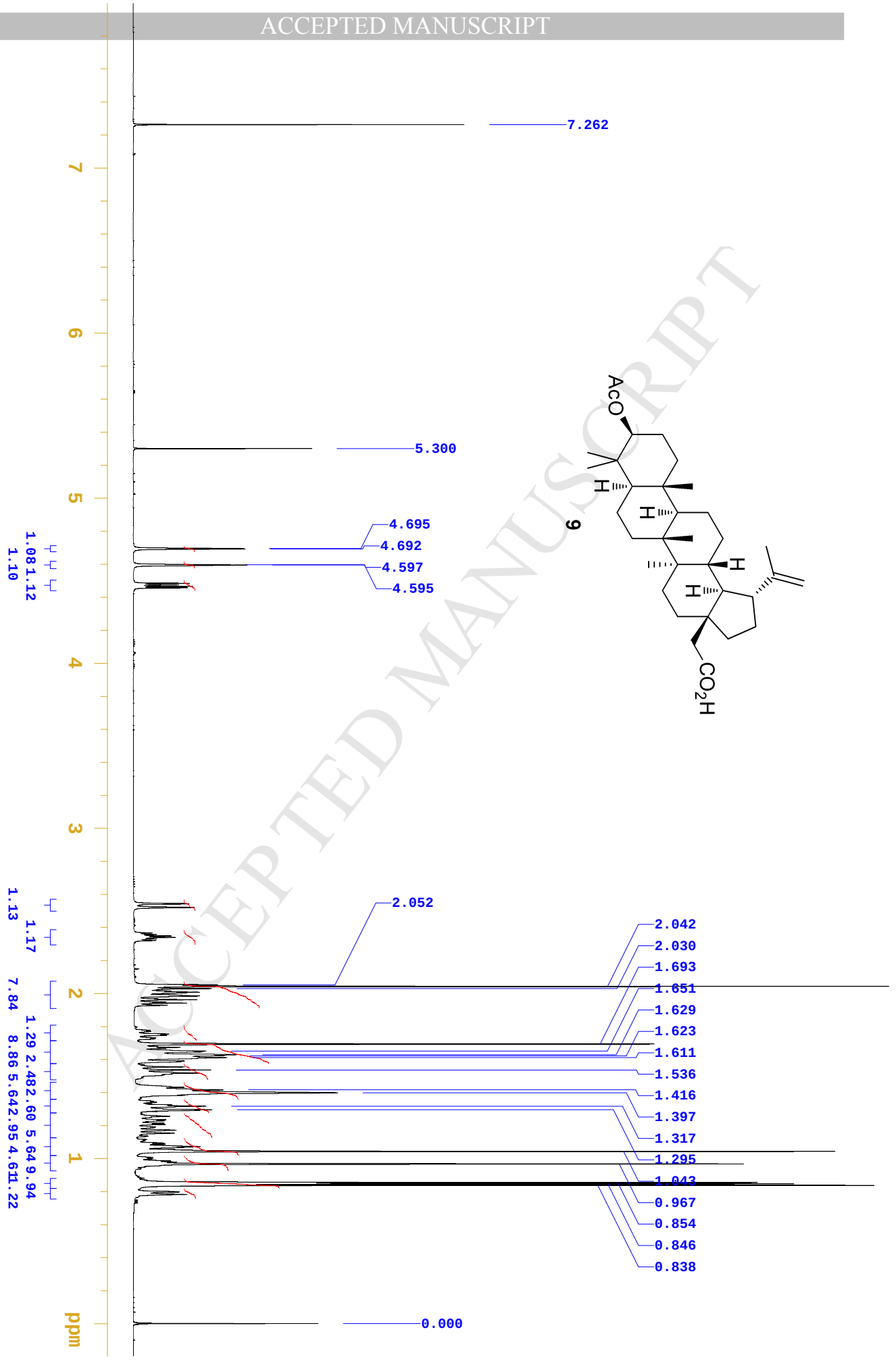

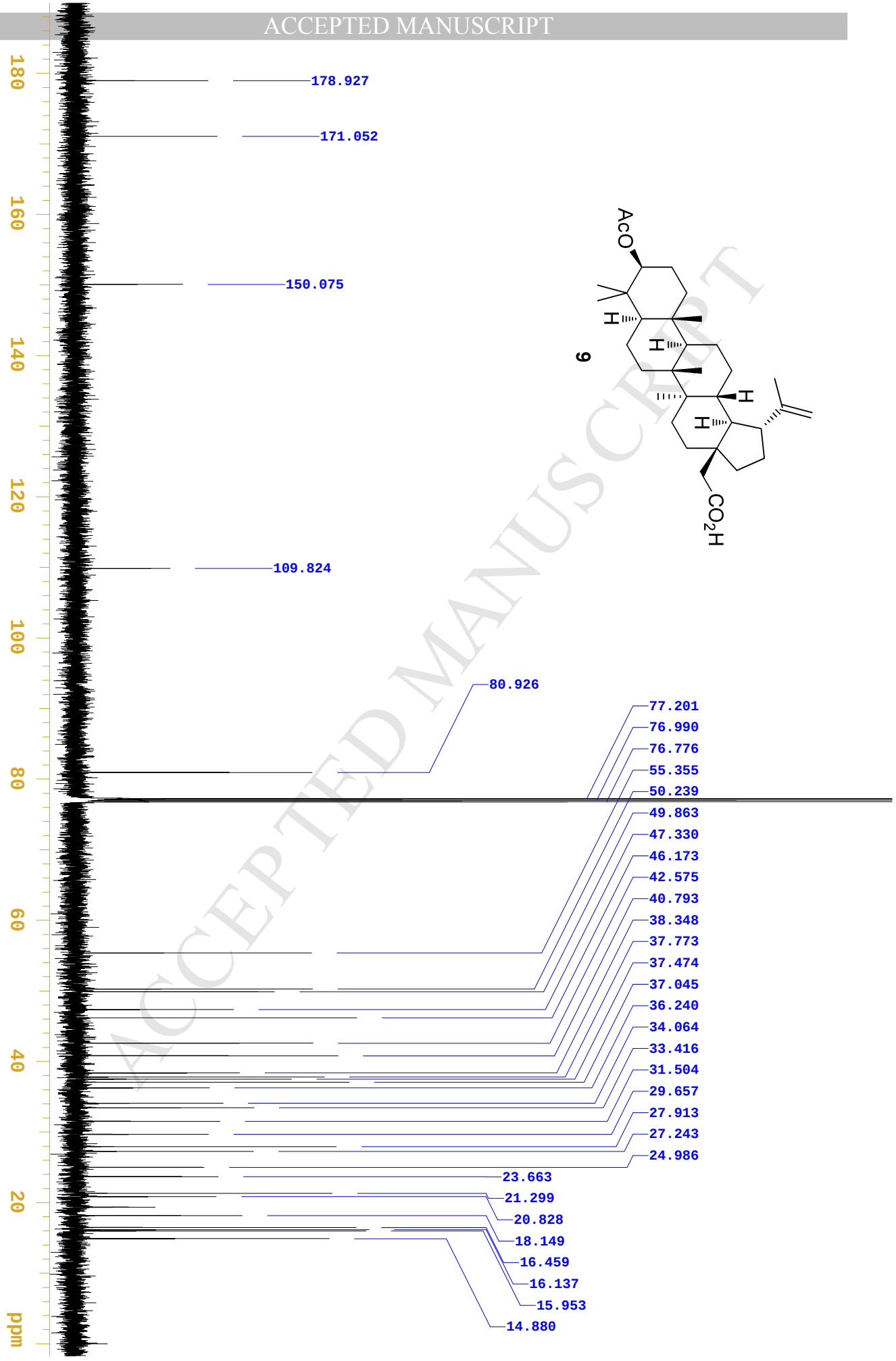

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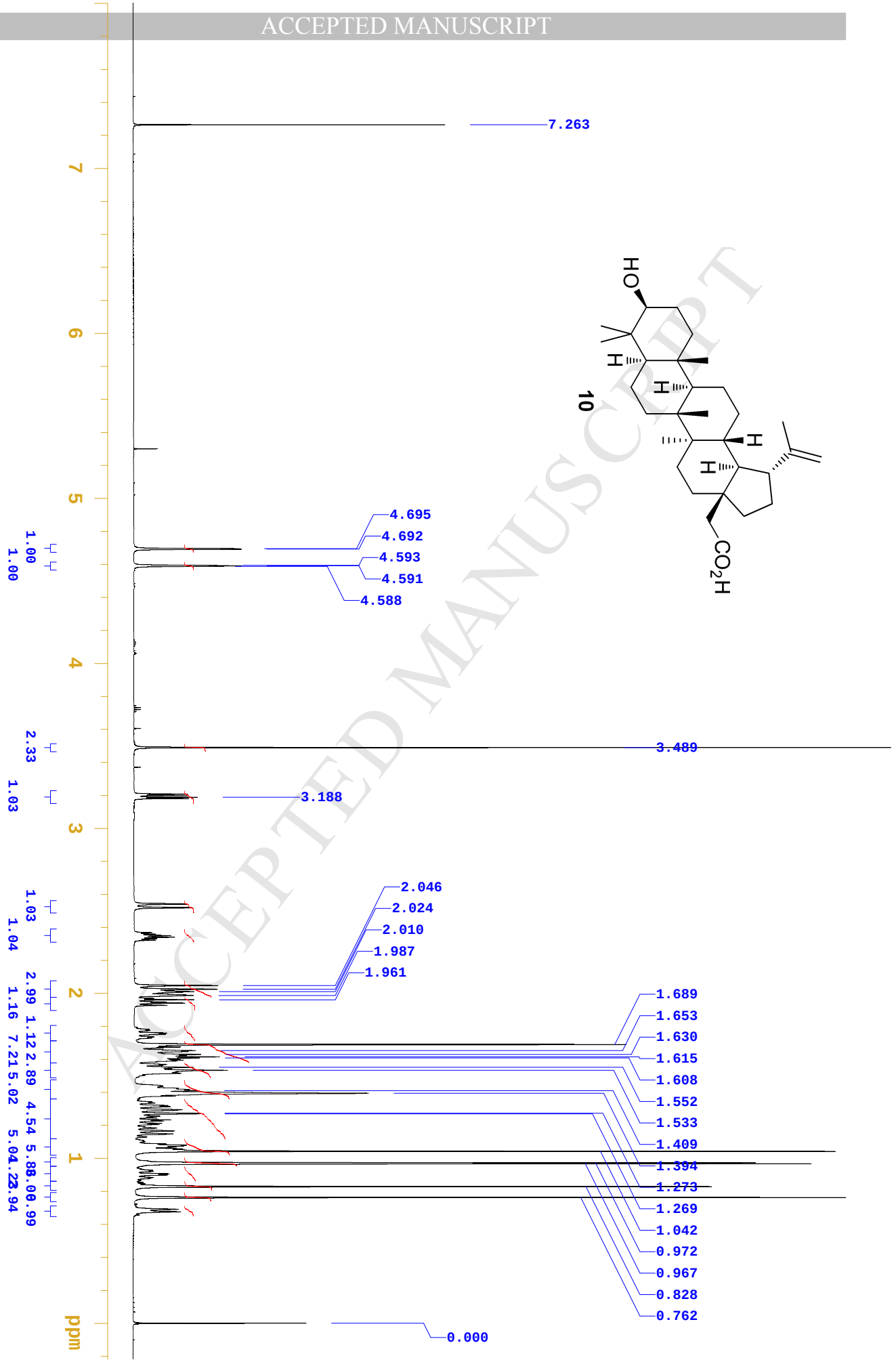

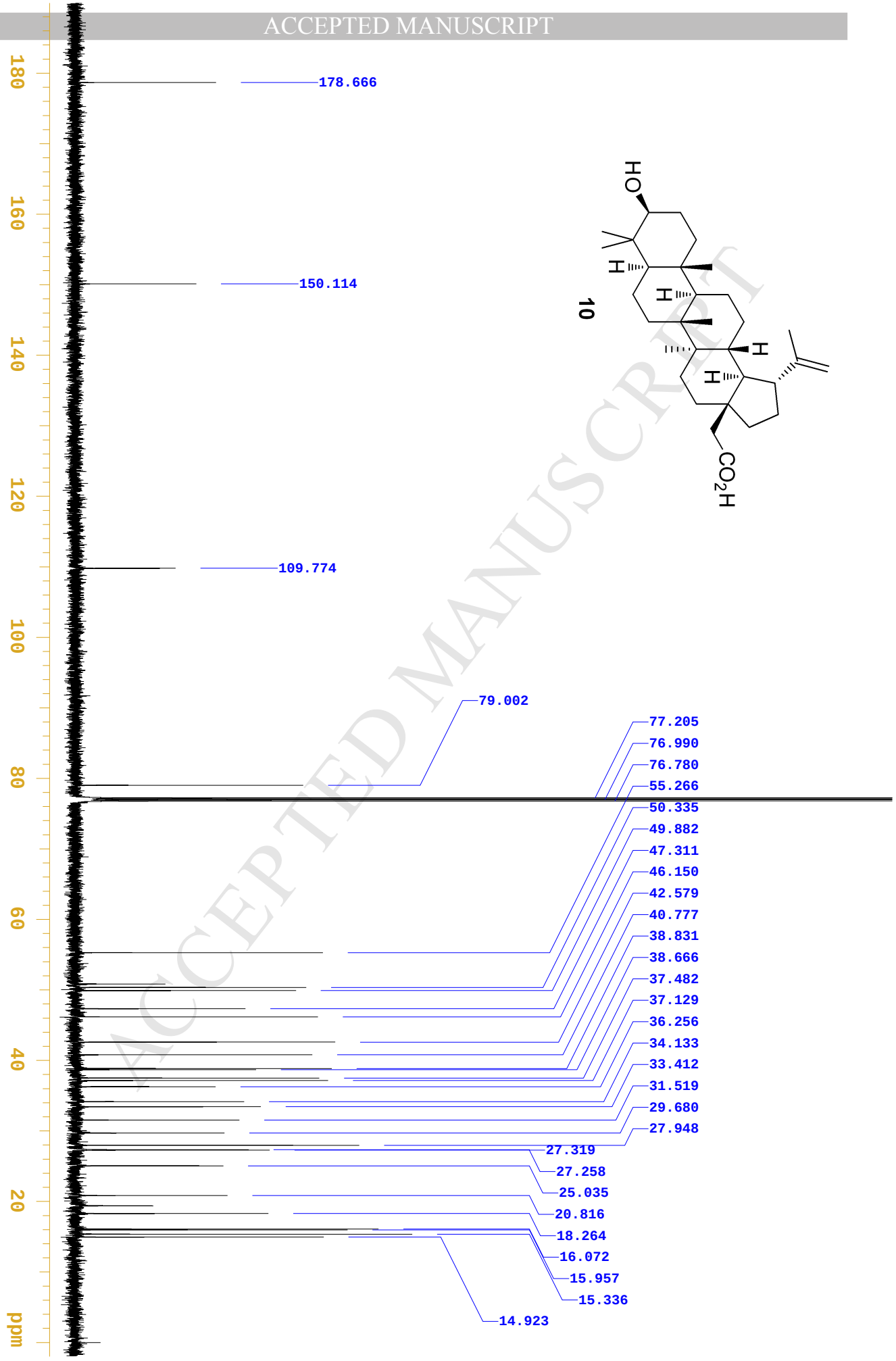

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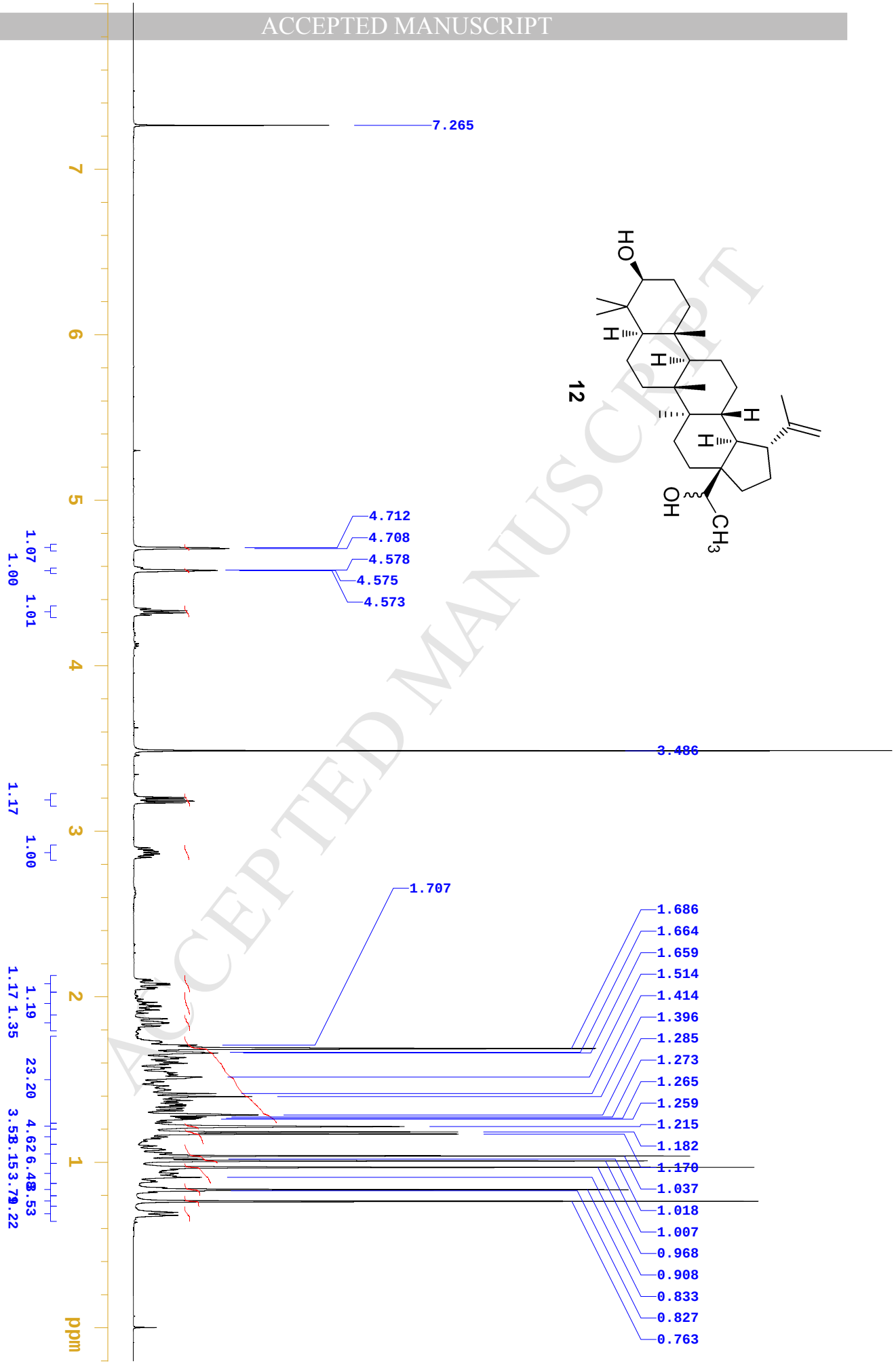

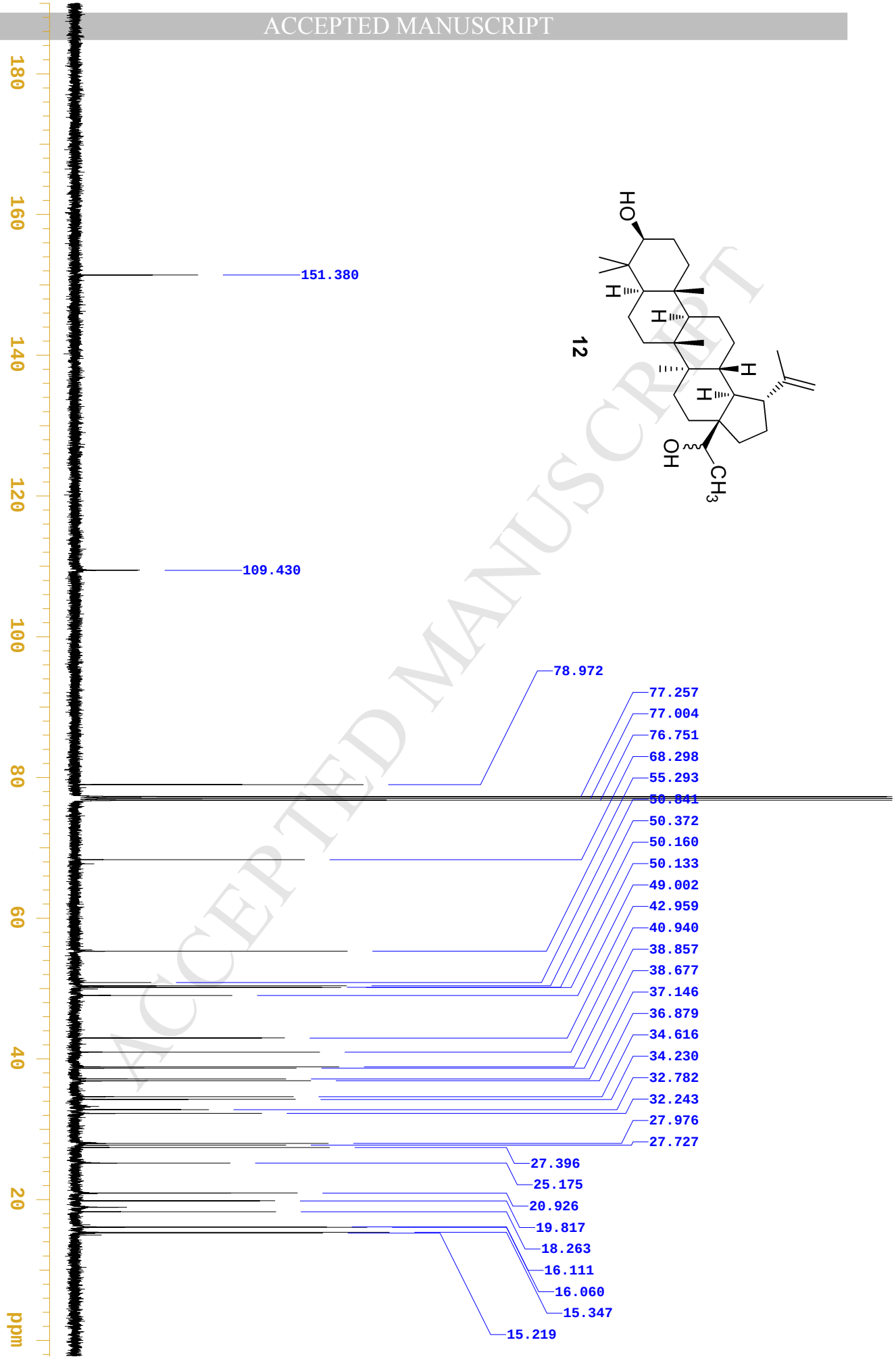

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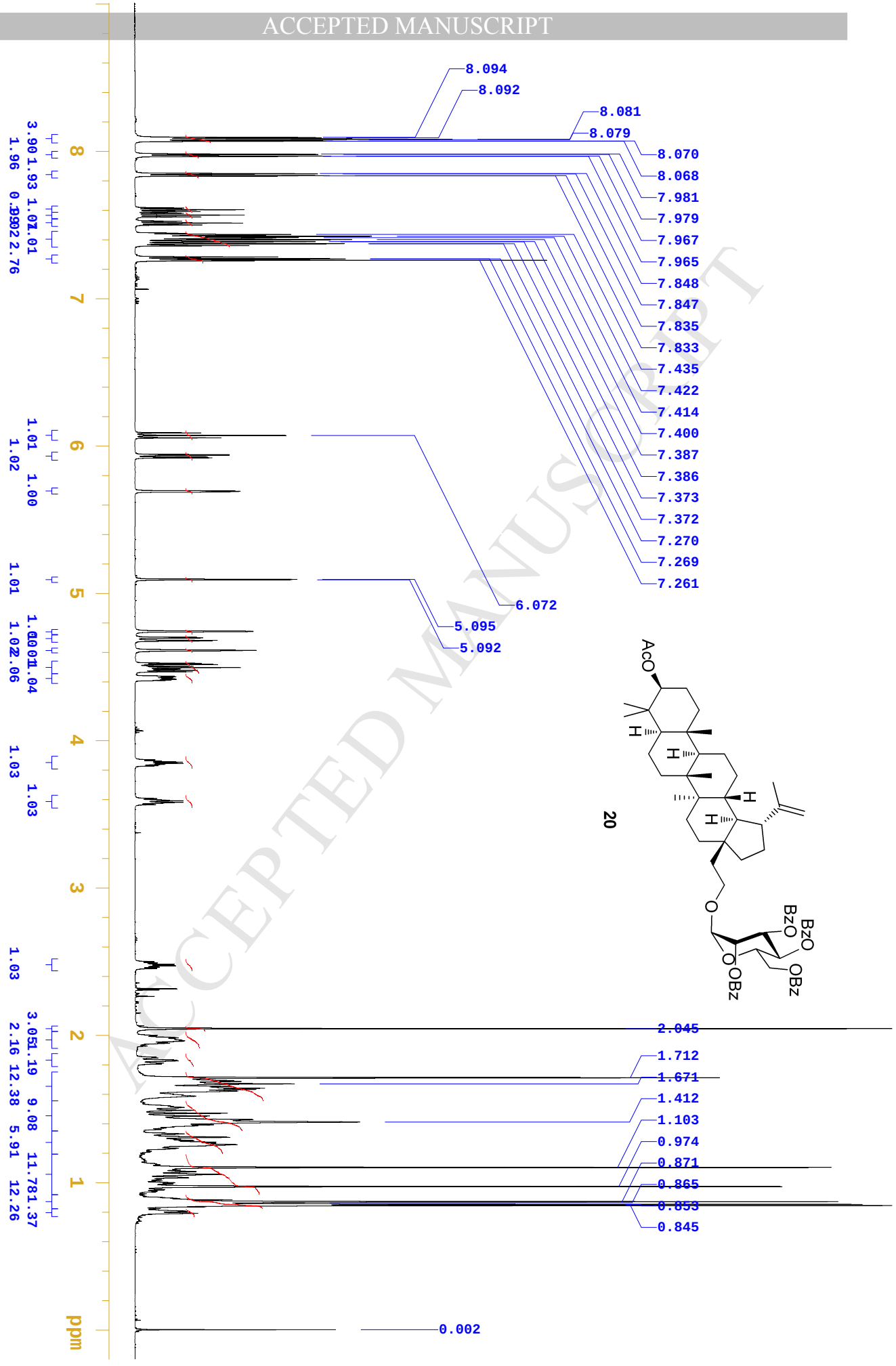

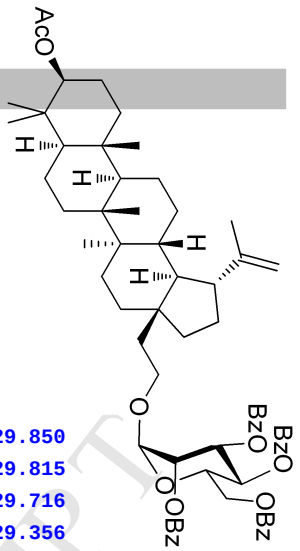

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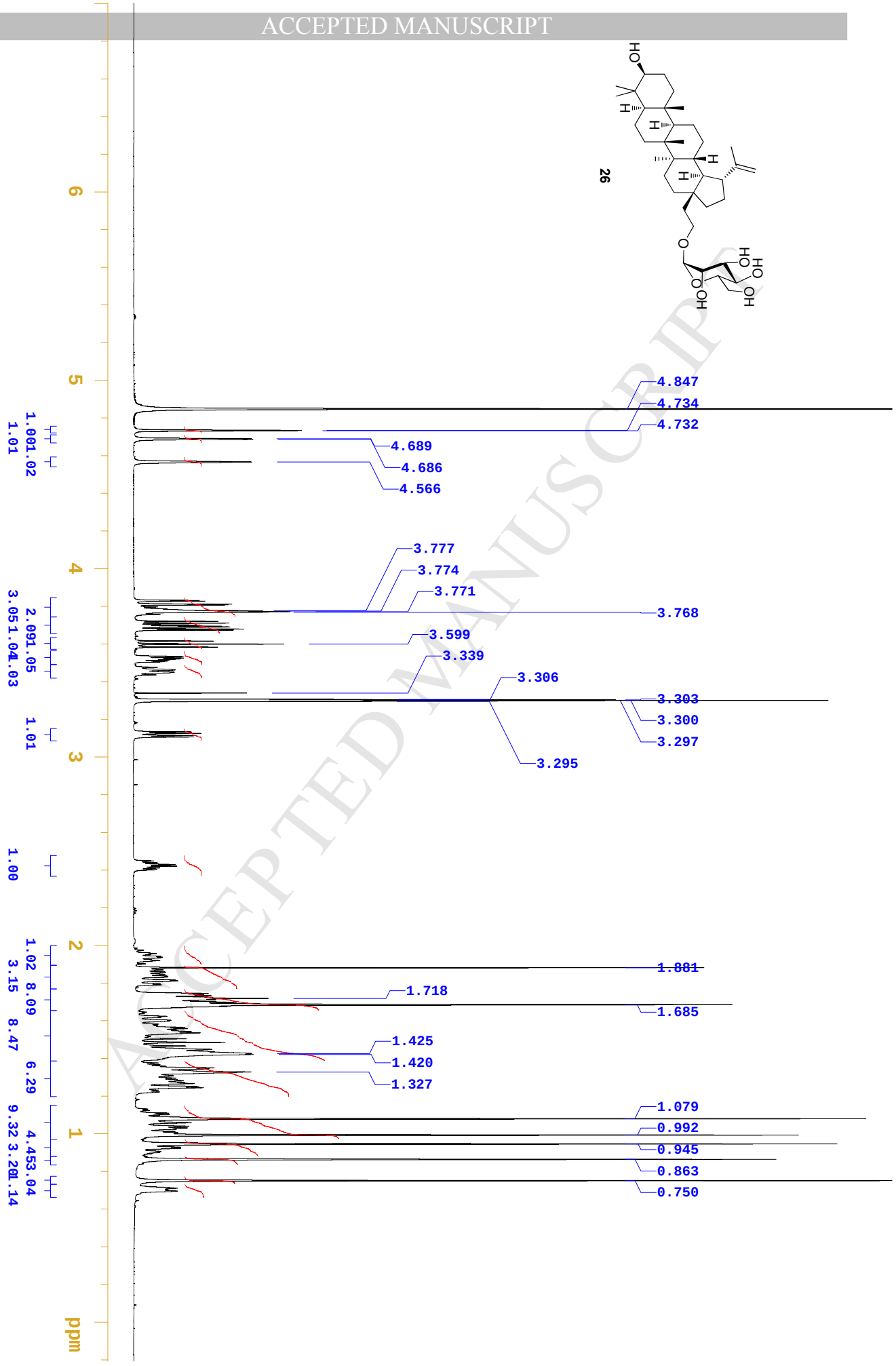
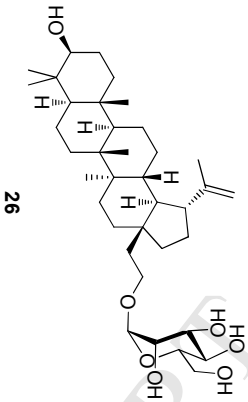
29.960
27.936
27.338
26.925
25.081
23.706
21.307
20.966
18.203
16.482
16.279
16.183
14.865

160
140
120
100
80
60
40
20
ppm

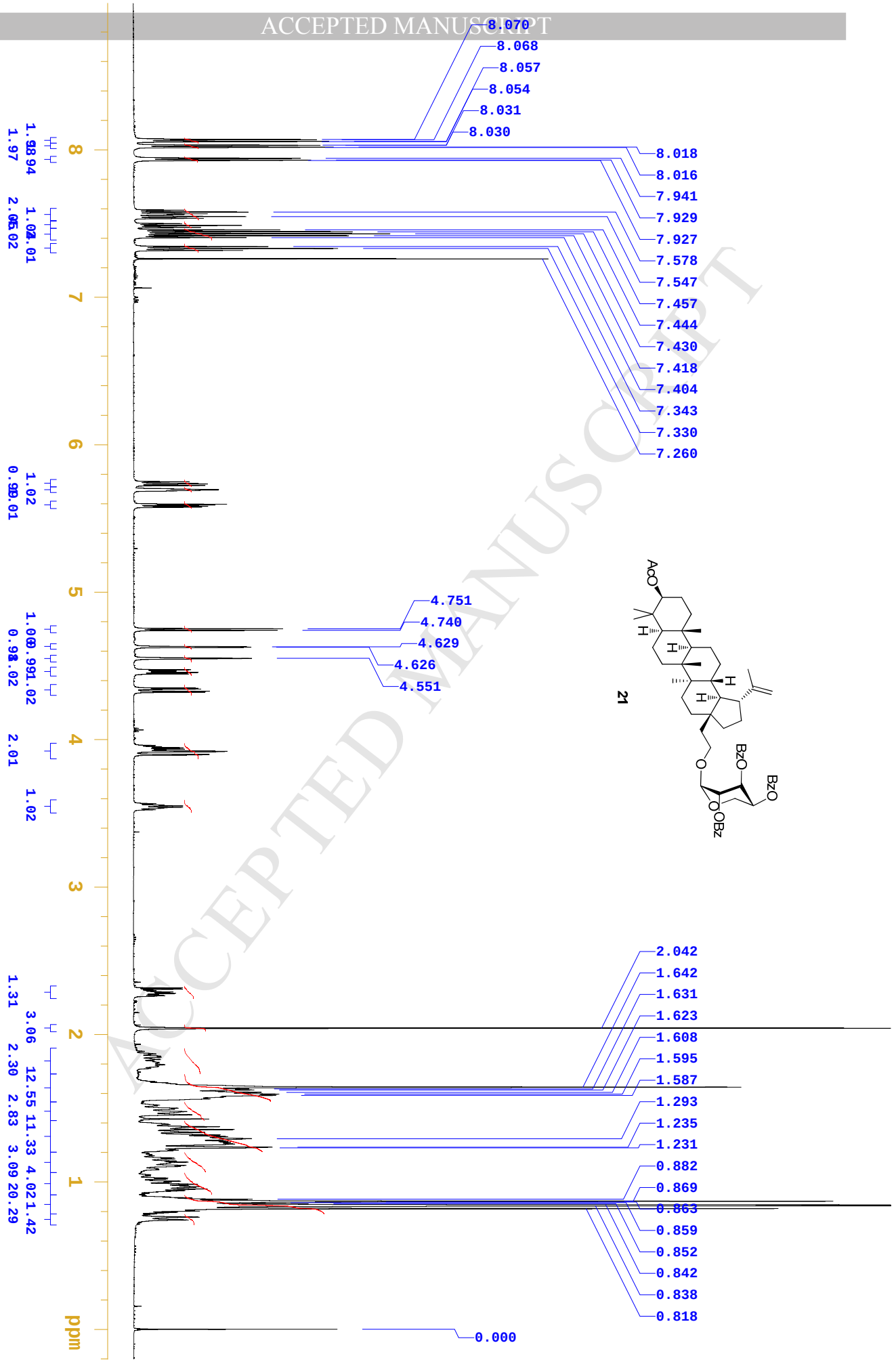


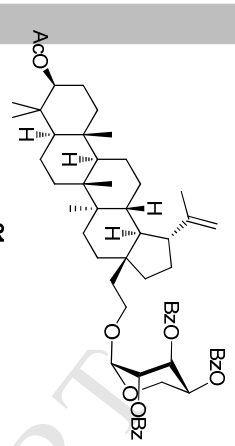


26

151.864
110.221
101.672
79.653
74.759
72.705
72.303
68.655
65.796
62.903
56.829
51.893
51.376
49.425
49.284
49.142
49.000
48.858
48.716
48.571
46.176
43.666
42.106
40.056
39.953
38.611
37.197
35.461
32.641
31.078
28.617
28.514
28.035
26.483
22.076
19.597
19.463
16.715
16.684
16.121
15.397

150 140 130 120 110 100 90 80 70 60 50 40 30 ppm





21

170.995
165.588
165.201
150.558
133.379
133.287
133.253
129.903
129.842
129.769
129.459
129.398
128.463
128.378
128.332
80.918
77.209
76.994
76.783
69.913
55.343
50.281
49.925
47.250
44.629
42.360
40.731
38.336
37.765
37.041
37.026
35.972
33.976
31.393
27.921
27.181
27.024
24.955
23.671
21.303
20.870
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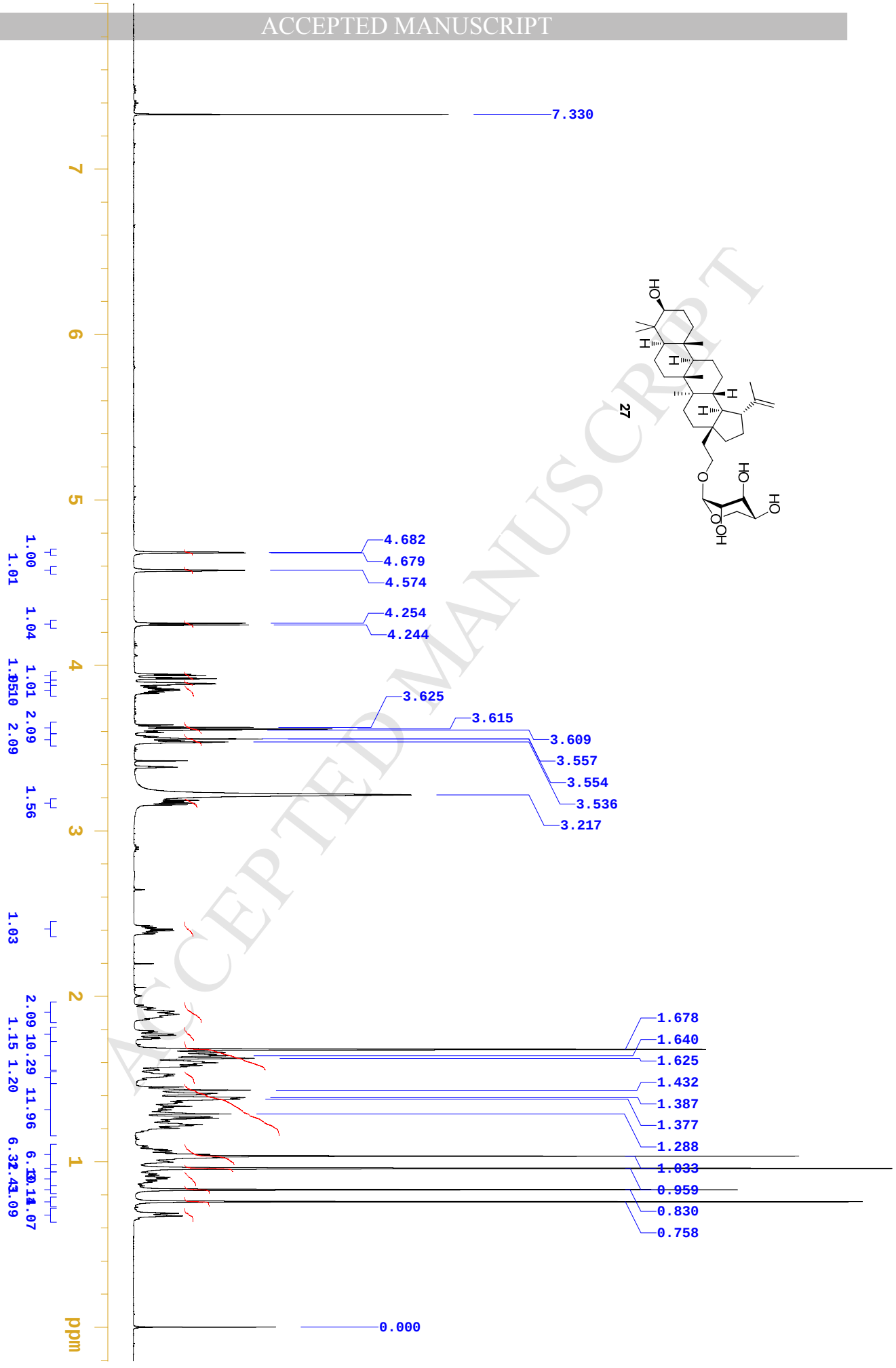
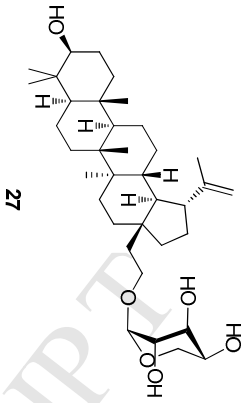
16.475
16.130
15.800
14.785

160
140
120
100
80
60
40
20
ppm





27

150.524
102.669
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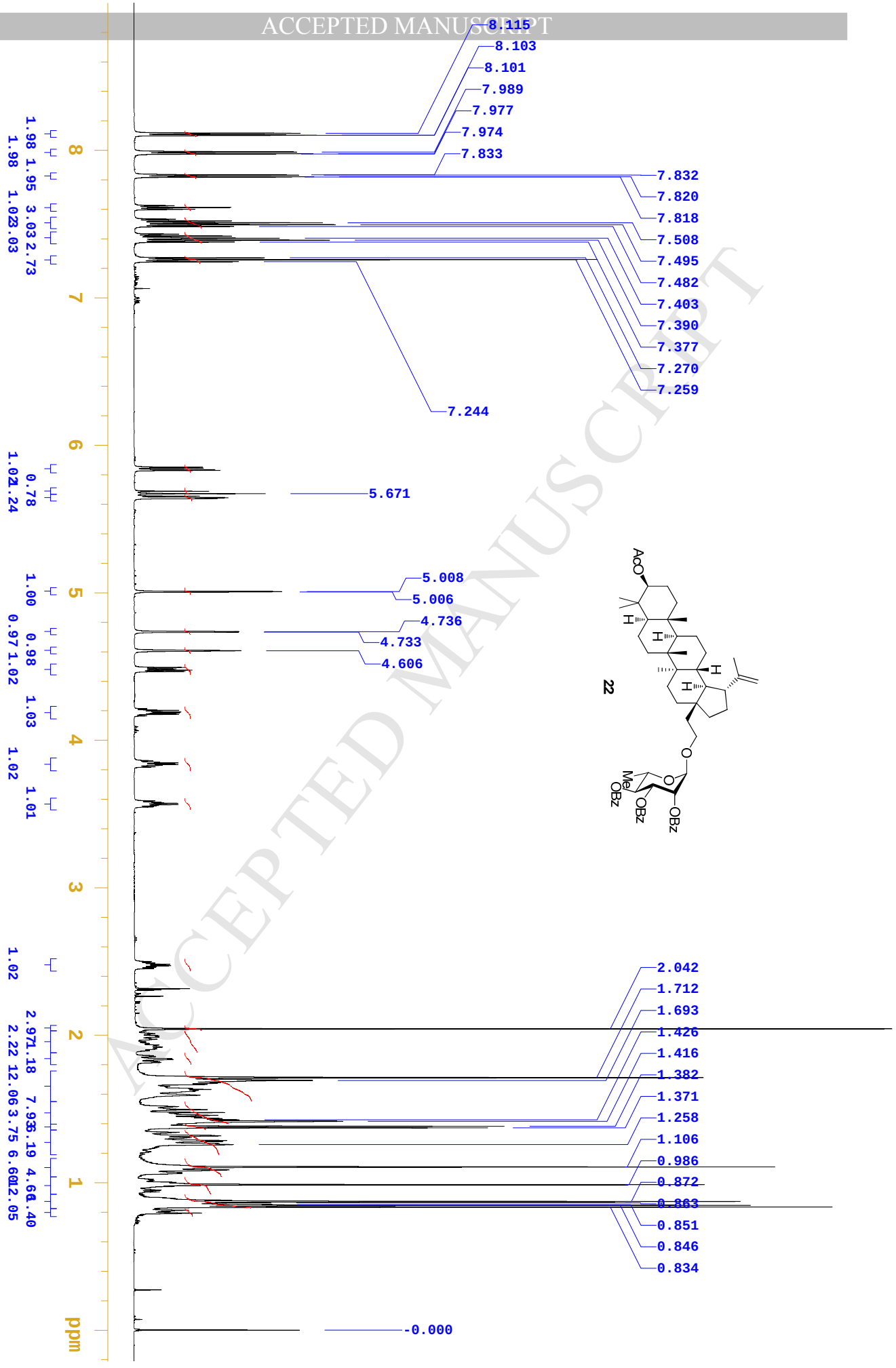
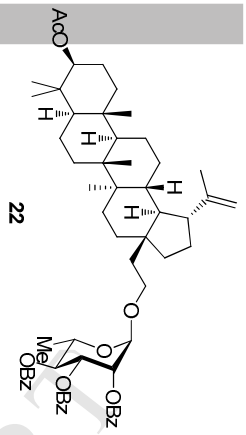
77.048
76.837
72.664
71.116
66.743
55.228
50.350
49.974
49.388
49.246
49.105
48.963
48.817
47.254
44.594
42.418
40.754
38.723
38.635
37.095
37.045
35.938
34.144
31.331
29.825
27.798
27.216
26.963
26.721
24.978
20.824
18.191
15.973
15.911
15.244
14.769

160
140
120
100
80
60
40
20
ppm





22

171.013
165.806
165.660
165.480
133.448
133.298
133.053
129.930
129.754
129.704
129.501
129.386
129.290
128.581
128.420
128.259
97.507
80.956
77.235
77.021
76.810
71.958
71.046
70.046
66.800
65.524
55.412
50.399
50.112
47.387
44.785
42.570
40.938
38.401
37.811
37.259
37.117
36.125
34.182
31.569
27.951
27.369
27.093
23.724
21.329
20.996
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17.746
16.493
16.210
14.918

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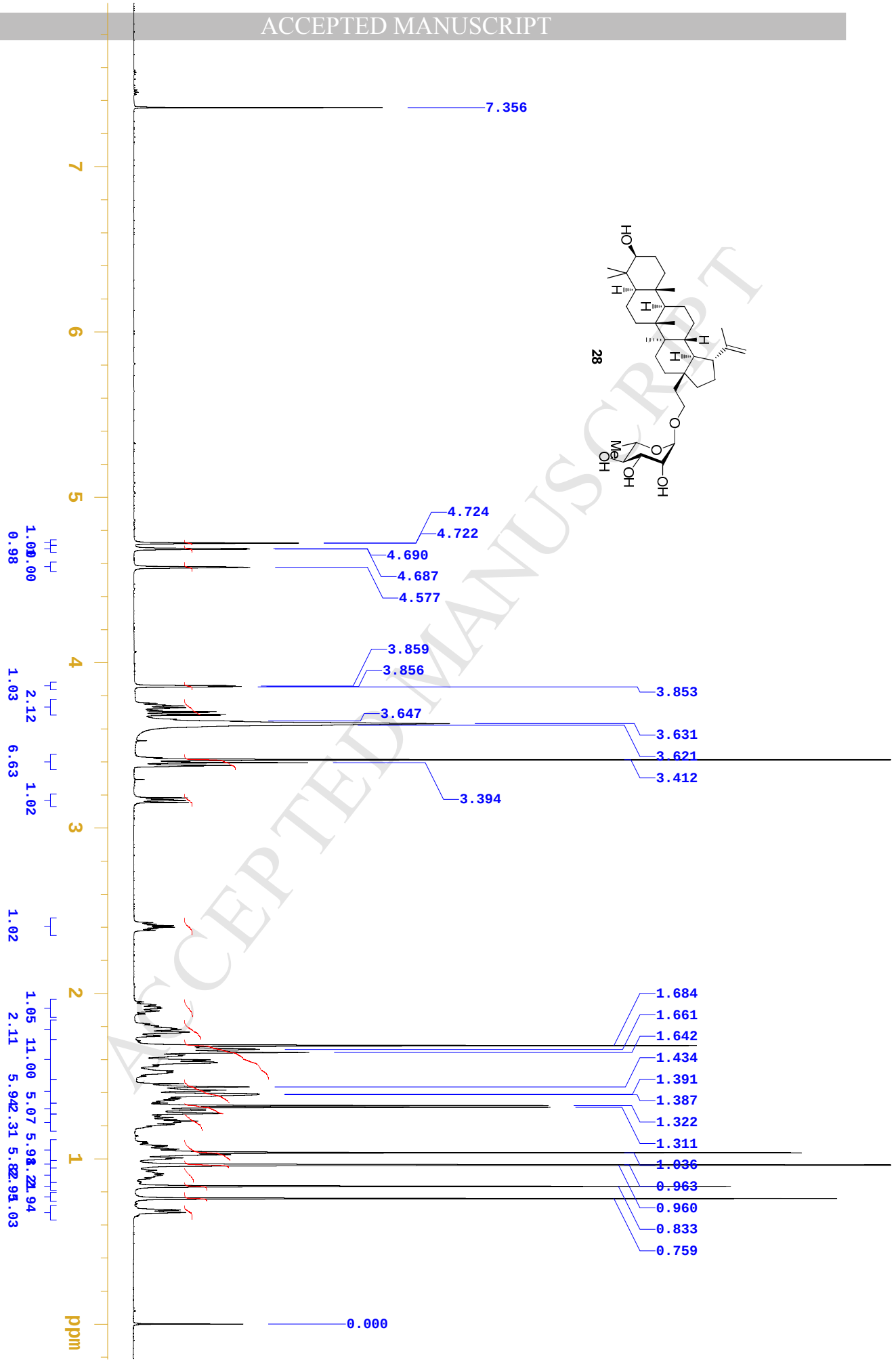
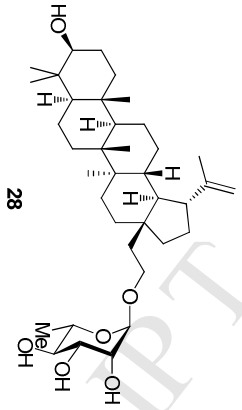
140
120
100
80
60
40
20
ppm





28

150.633
109.454
99.897
78.774
77.357
77.146
76.931
72.873
71.493
70.819
68.117
64.795
55.299
50.413
50.038
49.862
49.428
49.287
49.145
48.999
48.858
48.716
48.574
47.348
44.711
42.462
40.795
38.772
38.706
37.147
37.093
36.009
34.188
31.460
29.904
27.823
27.237
26.961
26.888
25.053
20.887
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15.998
15.952
15.285
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140
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60
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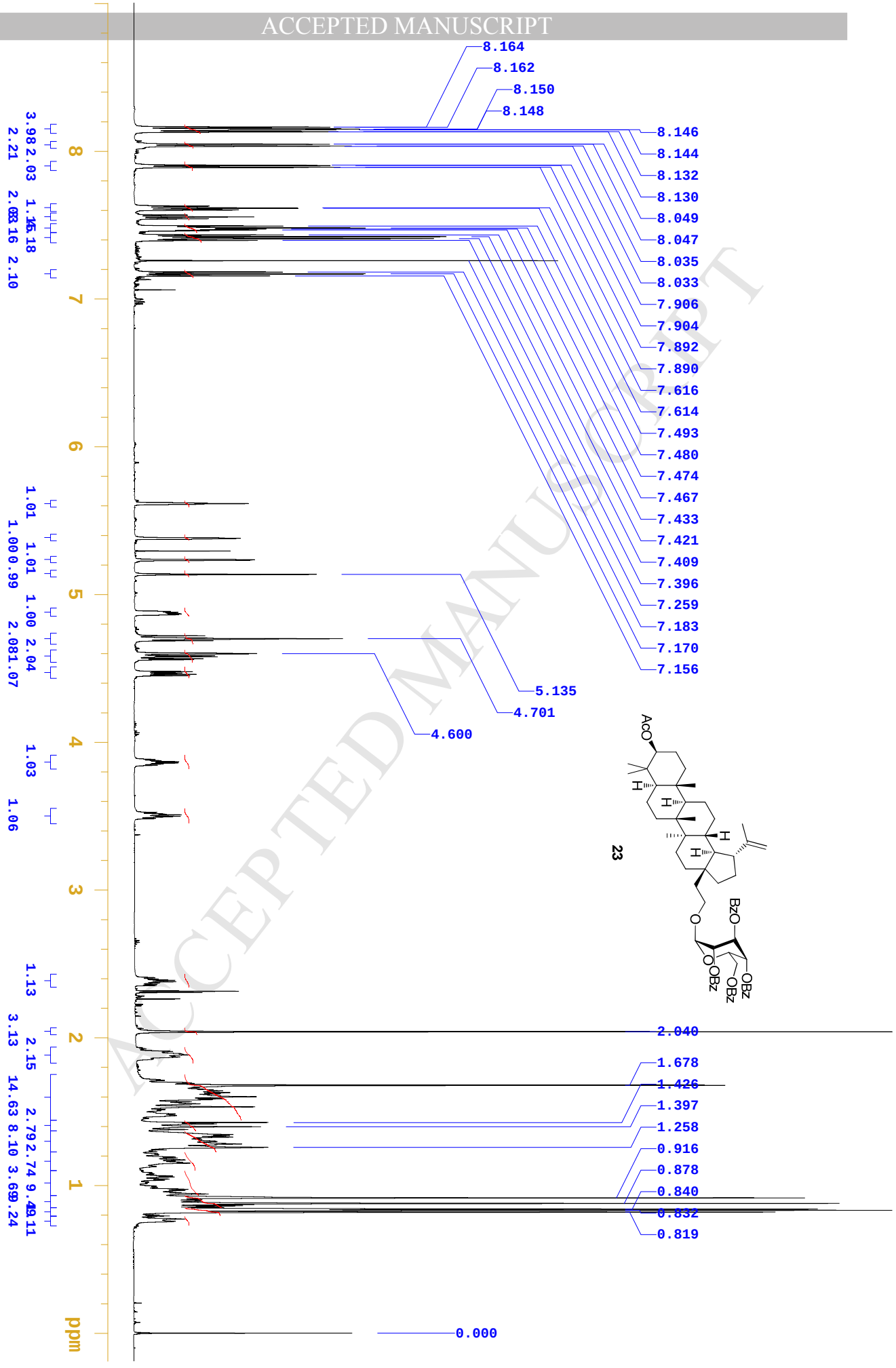
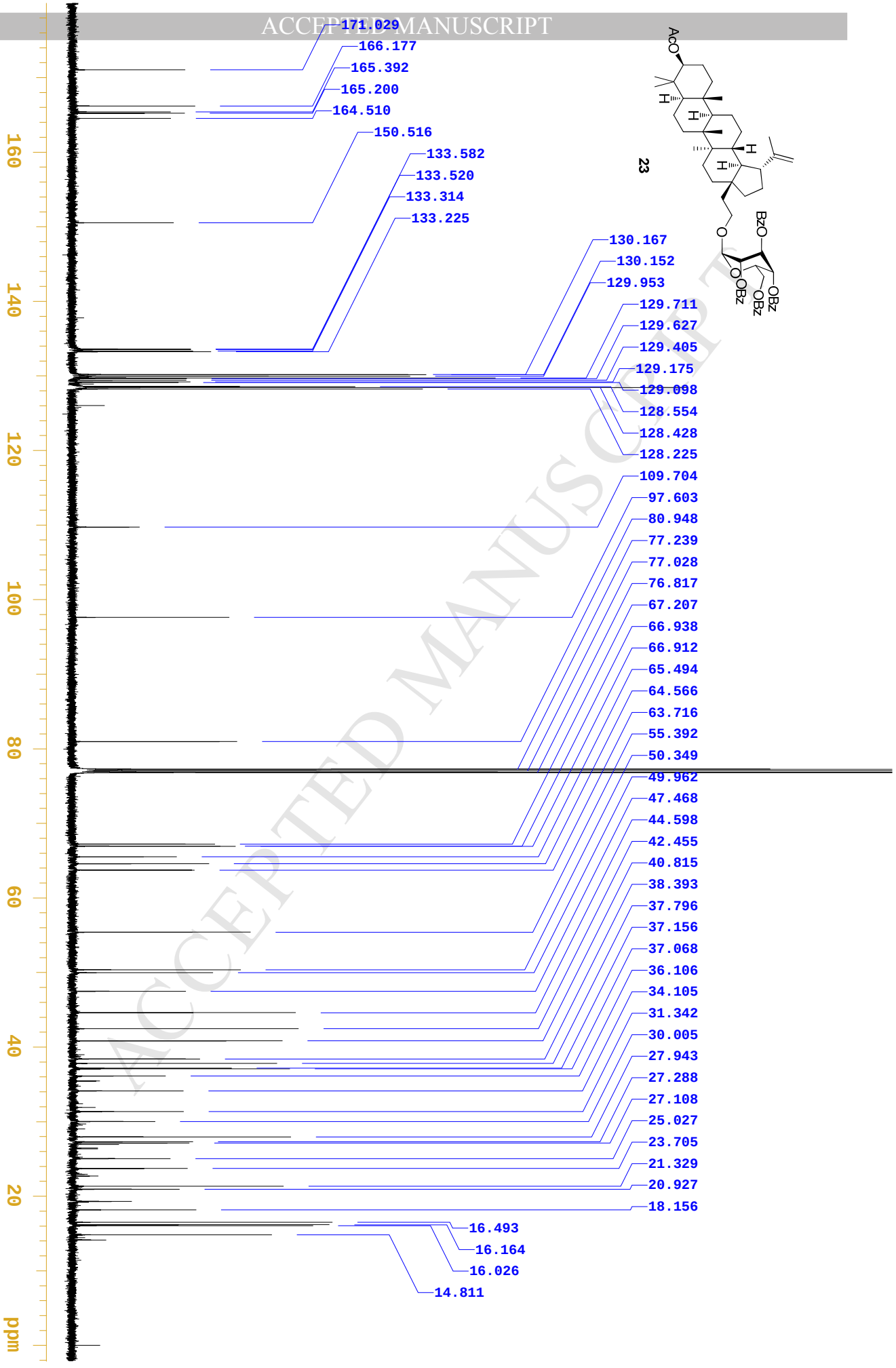
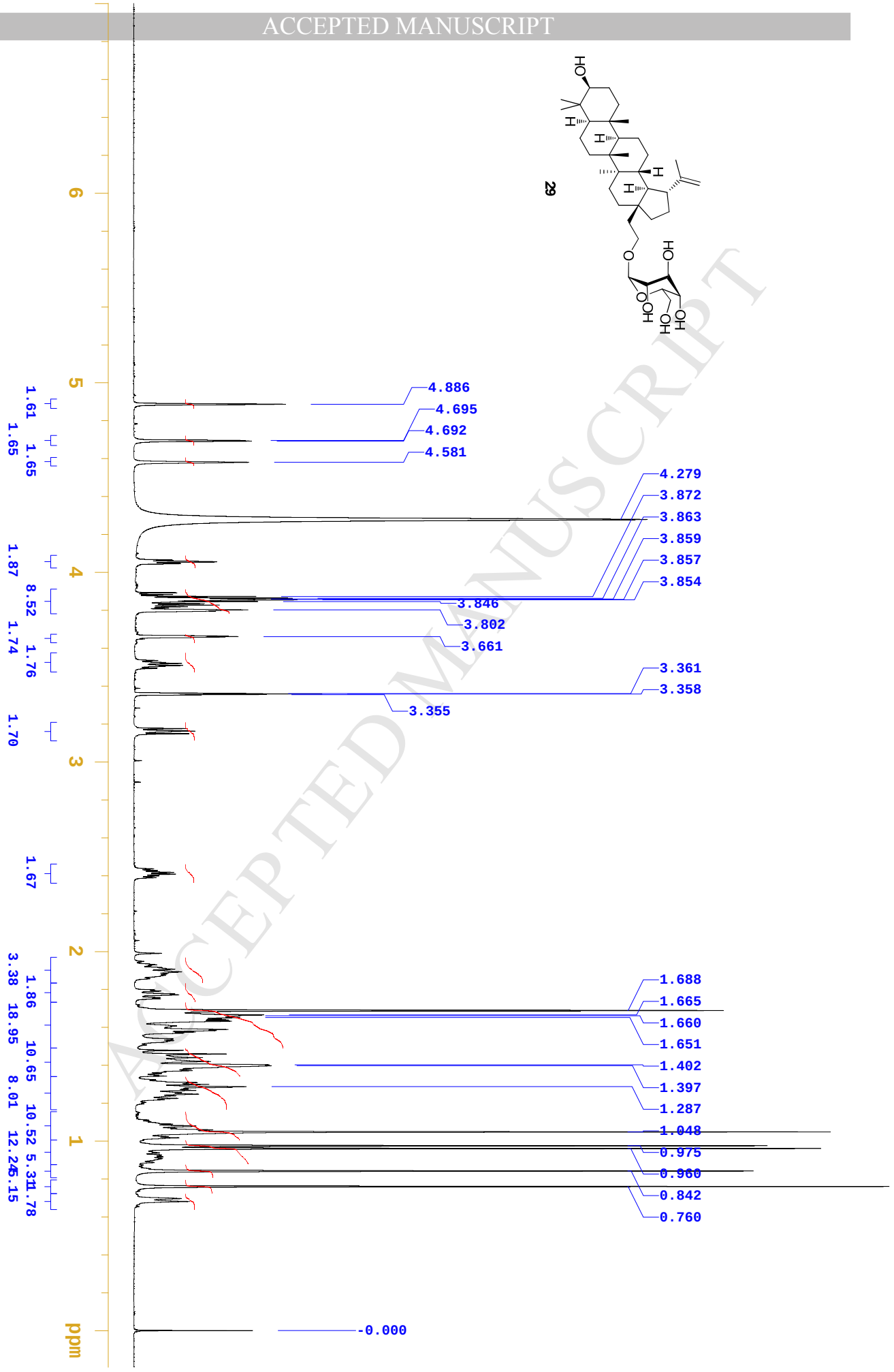
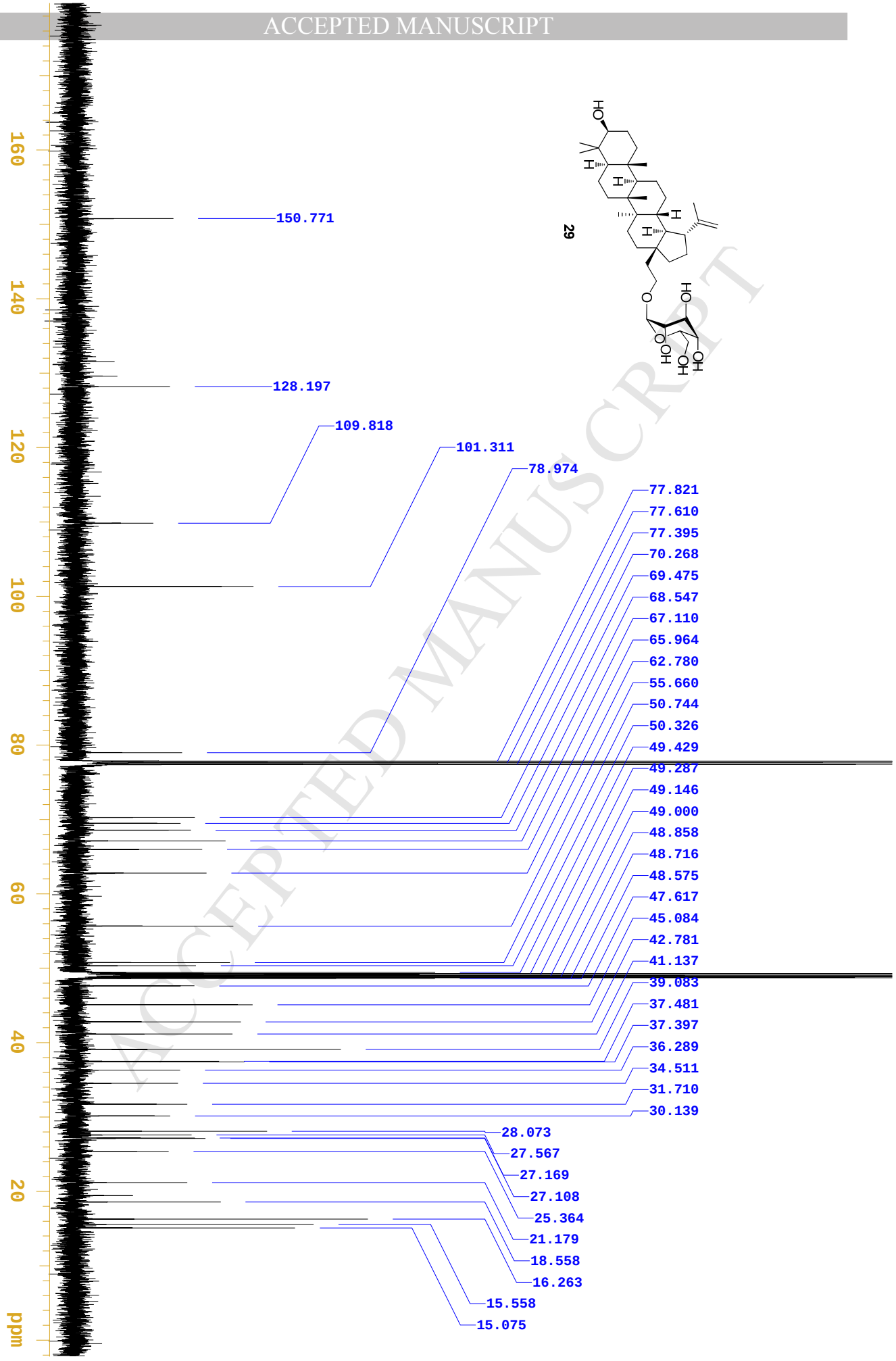
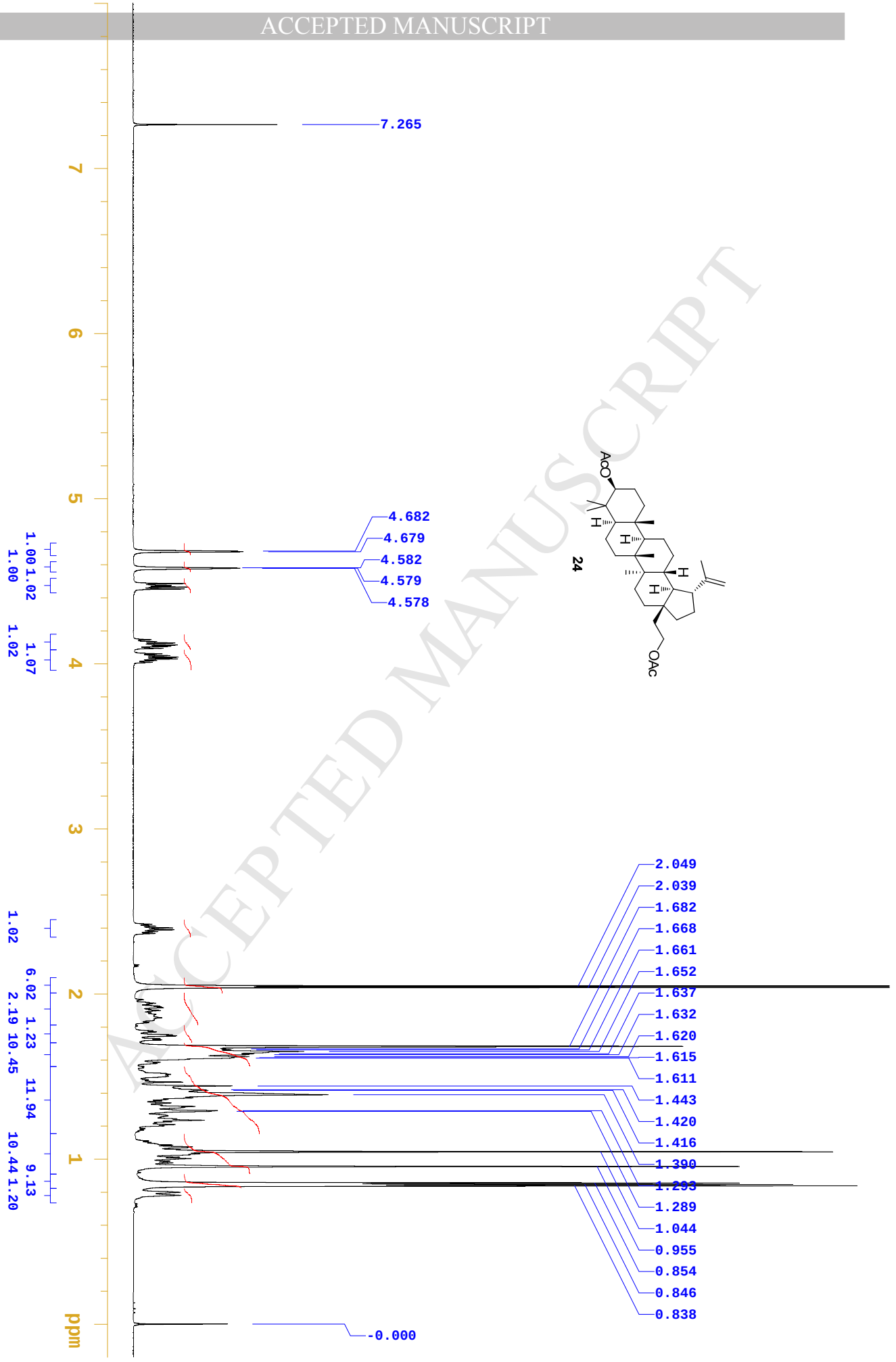
20
ppm

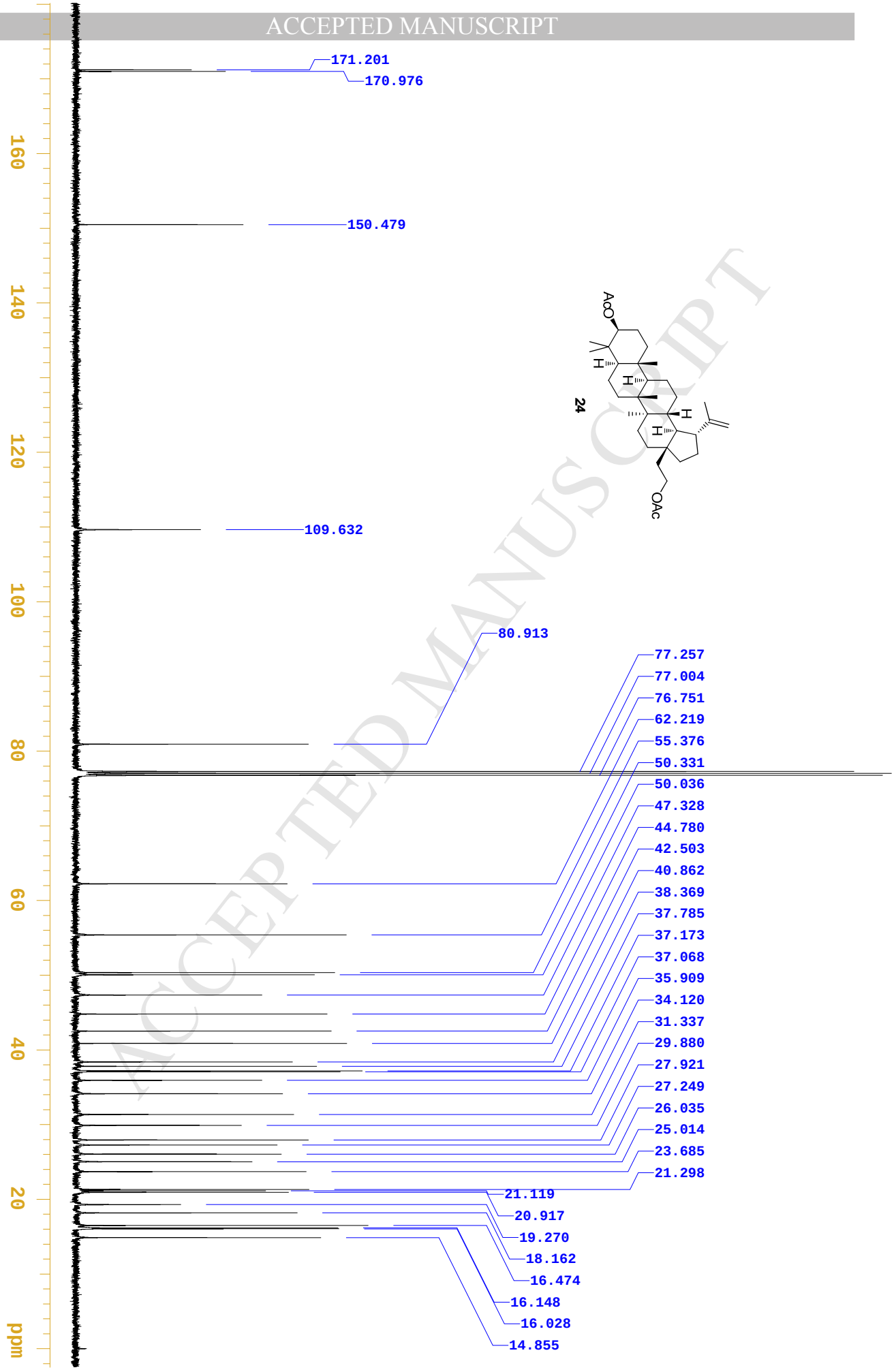












24