Mannose-Pepstatin Conjugates as Targeted Inhibitors of Antigen Processing

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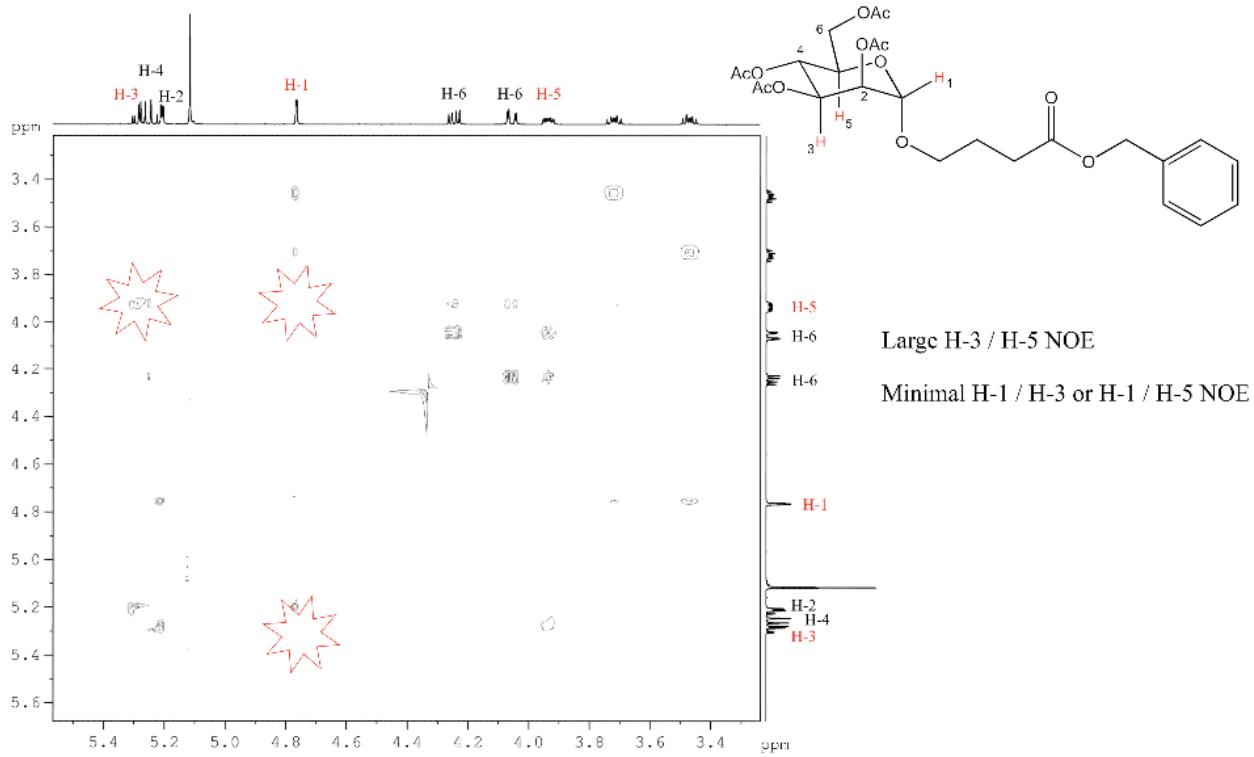
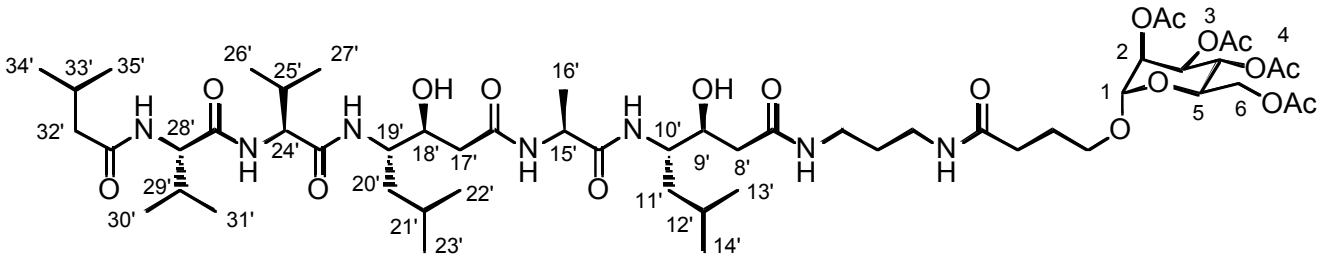
Supporting Information

Appendix 1: NOE of 3-benzyloxycarbonylpropyl 2,3,4,6-tetra-O-acetyl-α-D-mannopyranoside



Assignment of ^{1}H and ^{13}C chemical shifts for the acetylated mannose-pepstatin conjugate 13



Description	Number	^{13}C ppm	^{1}H ppm
Alanine CH_3	16’	18.08	1.37 (d, *J* = 7.24 Hz, 3H, H-16’)
CH_3	13’, 14’, 22’, 23’, 26’, 27’, 30’, 31’, 34, 35’	18.90, 19.11, 19.95, 20.05, 22.39, 22.40, 22.77, 22.81, 23.73, 23.81	*0.84 - 0.98 (m, 10 x 3H, H-13’, H-14’, H-22’, H-23’, H-26’, H-27’, H-30’, H-31’, H-34’ & H-35’)*
Acetyl		20.59, 20.64, 20.67	1.95, 2.03, 2.05 and 2.13 (s, 4 x 3H)
Statine γ-CH	12’, 21’	25.84, 25.91	1.54 - 1.65 (m, 4H, *H-12’, H-21’* and [H-11’ or H-20’])
CH_2	2’	26.60	1.93 - 1.95 (m, 2H, H-2’)
Isovaleryl CH	33’	27.48	2.02 - 2.10 (m, 3H, H-25’, H-29’ & *H-33’*)
Diamine CH_2-$\underline{CH_2}$-CH_2	5’	30.09	1.69 (quin, *J* = 6.80 Hz, 2H, H-5’)
Valine β-CH	25’, 29’	31.43, 31.54	2.02 - 2.10 (m, 3H, *H-25’, H-29’* & H-33’)
Propyl CH_2-C=O	3’	33.75	2.31 (t, *J* = 7.41 Hz, 2H, H-3’)
Diamine CH_2-NH	4’, 6’	37.85	3.17 - 3.26 (m, 4H, H-4’ & H-6’)
Statine CH_2	8’, 11’, 17’, 20’	41.26, 41.40, 41.65, 41.96	2.27 (d, *J* = 6.68 Hz, 2H, H-8’ or H-17’) 2.34 - 2.36 (m, 2H, H-8’ or H-17’)1.30 - 1.36 (m, 2H, H-11’ or H-20’) & 1.54 - 1.65 (m, 4H, H-12’, H-21’ and *[H-11’ or H-20’]*)
Isovaleryl CH_2	32’	46.0	2.11 - 2.14 (m, 2H, H-32’)
-OH		49.85	3.34 (s)
Alanine CH	15’	51.35	4.23 - 4.28 (m, 2H, H-6b & *H-15’*)
Statine α-CH	10’, 19’	52.33, 52.76	3.90 - 3.96 (m, 2H, H-10’, H-19’)
-OH		54.80	5.48 (s)
Valine α-CH	24’, 28’	60.75, 60.88	4.13 (d, *J* = 7.79 Hz, 1H, H-24’ or H-28’)

			4.15 (d, *J* = 8.05 Hz, 1H, H-24' or H-28')
Mannose CH_2	6a, 6b	63.61	4.09 (dd, *J* = 2.48, 12.24 Hz, 1H, H-6a) 4.23 - 4.28 (m, 2H, *H-6b* & H-15')
Mannose CH-4	4	67.81	5.20 - 5.24 (m, 3H, H-2, H-3, *H-4*)
Propyl CH_2-O-	1'a, 1'b	68.56	3.51 (ddd, *J* = 5.85, 6.39, 9.80 Hz, 1H, H-1'a) 3.75 (ddd, *J* = 5.99, 6.61, 9.80 Hz, 1H, H-1'b)
Mannose CH-5	5	69.84	3.98 - 4.03 (m, 3H, *H-5*, H-9' & H-18')
Mannose CH-2, CH-3 and statine CH-OH	2, 3, 9', 18'	70.73, 70.82, 71.11, 71.48	3.98 - 4.03 (m, 3H, H-5, H-9' & H-18') 5.20 - 5.24 (m, 3H, *H-2, H-3*, H-4)
Mannose CH-1	1	98.91	4.82 (d, *J* = 1.42 Hz, 1H, H-1)
Carbonyl		171.51, 171.52, 171.60, 172.40, 173.78, 173.92, 174.07, 174.16, 175.42, 175.44, 174.78	

The mannose-BSA conjugate 17 contains approximately 23 conjugated mannose sugars

MALDI-TOF mass spectroscopy gave a detectable average mass for BSA with a peak at 66,430 (Figure 1). The mass of BSA conjugate **17** gave a peak at 74,030 with a peak range between 67,000 – 79,000 (Figure 2). This range is expected due to the varied number of conjugated mannosyl compounds, with the median mass at 74,030. The difference between BSA and mannose-BSA conjugate **17** equates to approximately 24.3 mannose units / BSA molecule.

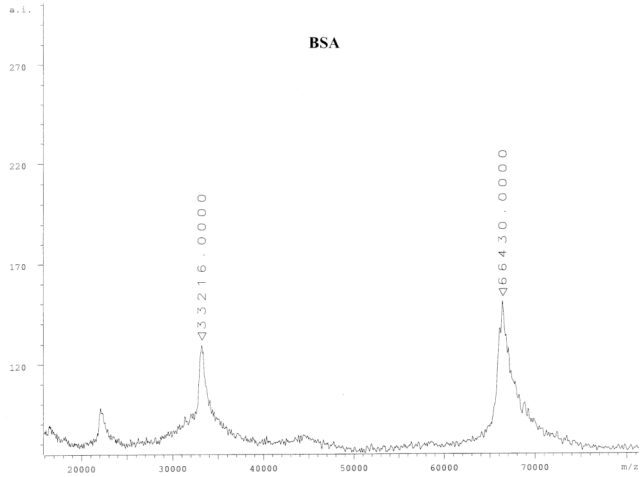


Figure 1: MALDI-TOF of BSA

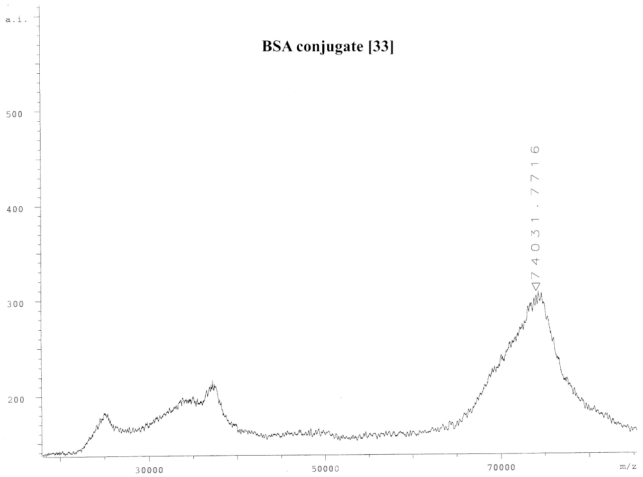


Figure 2: MALDI-TOF of mannose-BSA conjugate **17**

Biochemical analysis for the presence of sugars was carried out using the phenol – sulphuric acid method.[1] A graph of concentration of 4-aminophenyl-α-D-mannopyranoside *vs* absorption (Figure 3) was used as a reference for determining the amount of mannose sugars attached to mannose-BSA conjugate **17**. Table 1 shows the absorbance values obtained for mannose-BSA conjugate **17** (three different experiments, two concentrations per experiment) from which an estimated number of mannosyl units per BSA are calculated by using the linear equation derived from Figure 3. The mean of these gives a value of 23.1 +/– 2.5 mannosyl units per BSA for mannose-BSA conjugate **17**.

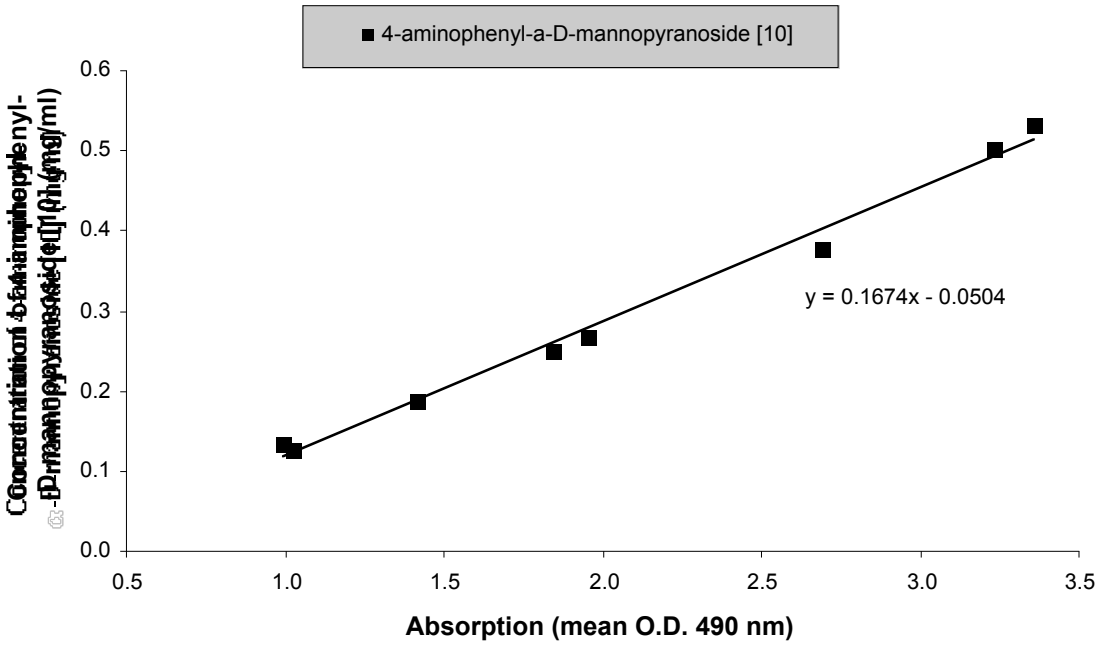


Figure 3: Selected data from a standard curve of 4-aminophenyl-α-D-mannopyranoside. Each data point is the mean of three separate experiments, each experiment being run in triplicate, and selected as all the data with absorbance between approx 1.0 and 3.5. This selected range fits a linear equation, the plot of which is used to help calculate the number of mannose units per mannose-BSA conjugate **17**.

Mannose-BSA conjugate 17 (mg/ml)	3.6	1.8	3.6	1.8	3.6	1.8
Absorbance (mean O.D. 490 nm)	2.15	1.18	1.79	1.35	2.19	1.22
Estimated number mannoses / BSA	23.52	22.31	18.89	26.62	23.97	23.24

Average	**S.D.**
23.1	2.5

Table 1: Estimated number of mannosyl sugar units per BSA molecule on mannose-BSA conjugate **17.** Absorptions of three separate tests of two different concentrations were run as triplicates, and from the absorption observed, the mean number of mannosyl sugars per BSA of mannose-BSA conjugate **17** was calculated from the equation derived from Figure Xc. The mean and standard deviation of these six values is calculated as 23.1 +/- 2.5

Biochemical analysis of the number of free lysine amino acids on BSA conjugate **17** was determined by the trinitrobenzenesulfonic acid (TNBS) method.[2,3] A standard curve of BSA and mannose-BSA conjugate **17** (Figure 4) was obtained by 1 in 2 dilutions from 10 mg/ml and 20 mg/ml of BSA and mannose-BSA conjugate **17** respectively. These plots gave a correlation that fits a quadratic equation (Figure 4). From this equation it was calculated that an absorbance = 1.0 is equivalent to 5.80 mg/ml (87.1 μM) of BSA and 10.44 mg/ml (141 μM) of mannose-BSA conjugate **17**.

To determine if all of the amino groups on BSA are able to react with TNBS, a test of the absorbance of BSA and BSA under denaturing conditions (addition of 10% of 2% w/v SDS) with a longer duration for TNBS reaction (30 mins compared with 5 mins normally) was performed both with and without a change of pH to neutrality after reaction (sometimes required to ensure non-reversible addition of TNBS addition by sulfite displacement). The results (not shown) showed a limited difference in the value obtained for the absorption. It was assumed that all of the amino groups of BSA were reactable towards TNBS (i.e. 60 including a terminal NH_2). Therefore:

Free amino groups per BSA = 60

87.1μM BSA x 60 amino groups = 5.23mM free amino groups per BSA

∴ 5.23mM / 141 μM mannose-BSA conjugate **17** concentration =

37 amino groups per BSA conjugate **17**

∴ 60 – 37 = <u>23 attached mannosyl units per mannosyl-BSA conjugate **17**</u>

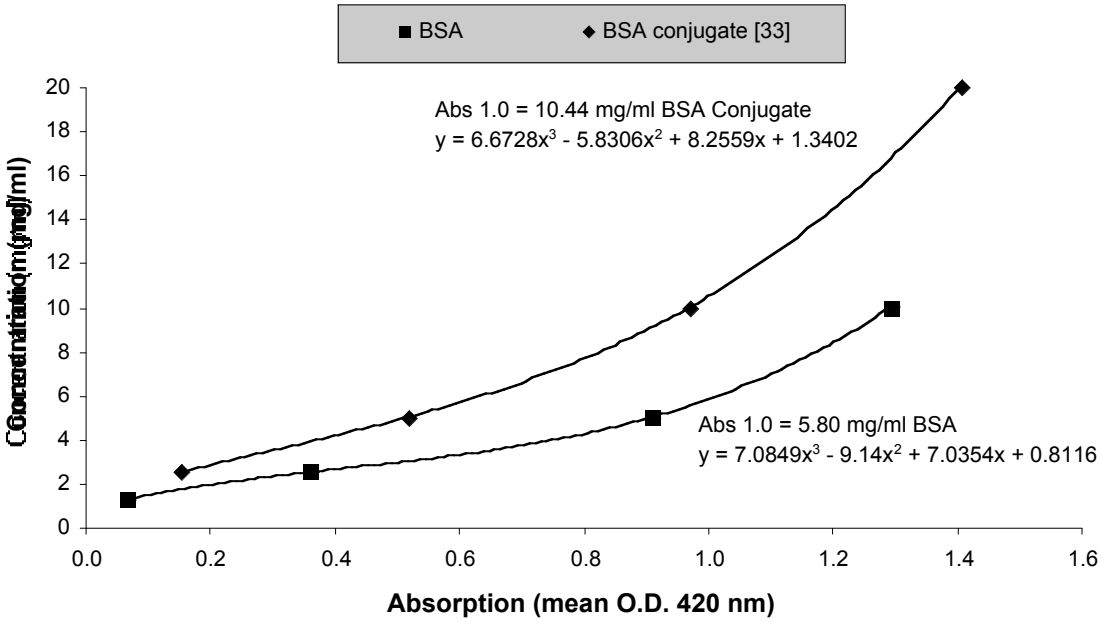


Figure 4: Standard curve of BSA and mannose-BSA conjugate **17**. Each data point is the mean of three separate experiments, each experiment being run in triplicate. Quadratic equations are included for each correlation, from which the number of free lysine amino groups on mannose-BSA conjugate **17** is calculated.

Mass spectroscopy confirms that 4 contains a single attached pepstatin

Determination of the protein sulfhydryl groups on neomannosylated BSA-pepstatin conjugates **3** and **4** with Ellman's reagent did not give reproducible results. However, MALDI-TOF mass spectroscopy confirmed that a single pepstatin molecule had been attached to BSA conjugate **17** to give conjugates **3** and **4**.

MALDI-TOF mass spectroscopy for **4** shows a peak range = 69,000 – 81,500 (Figure 5), with the median peak for **4** being ~ 75,163. This is an increase in median peak value over BSA conjugate **17** of 1,131 (pepstatin analogue MW = 861) and a peak range increase in mass of at least 2,000 MW. Both factors indicate the presence of an extra molecule.

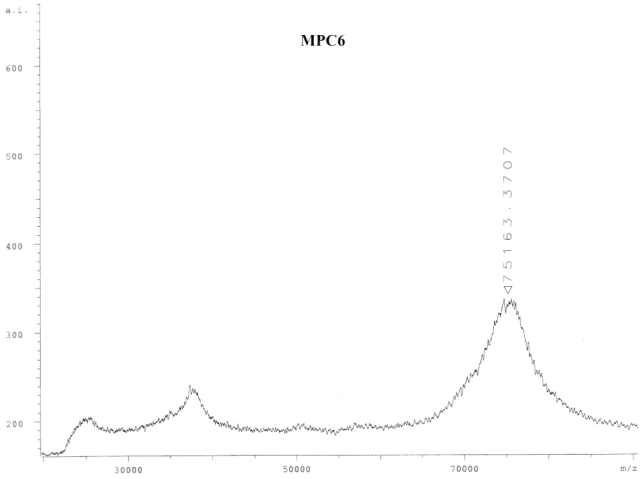


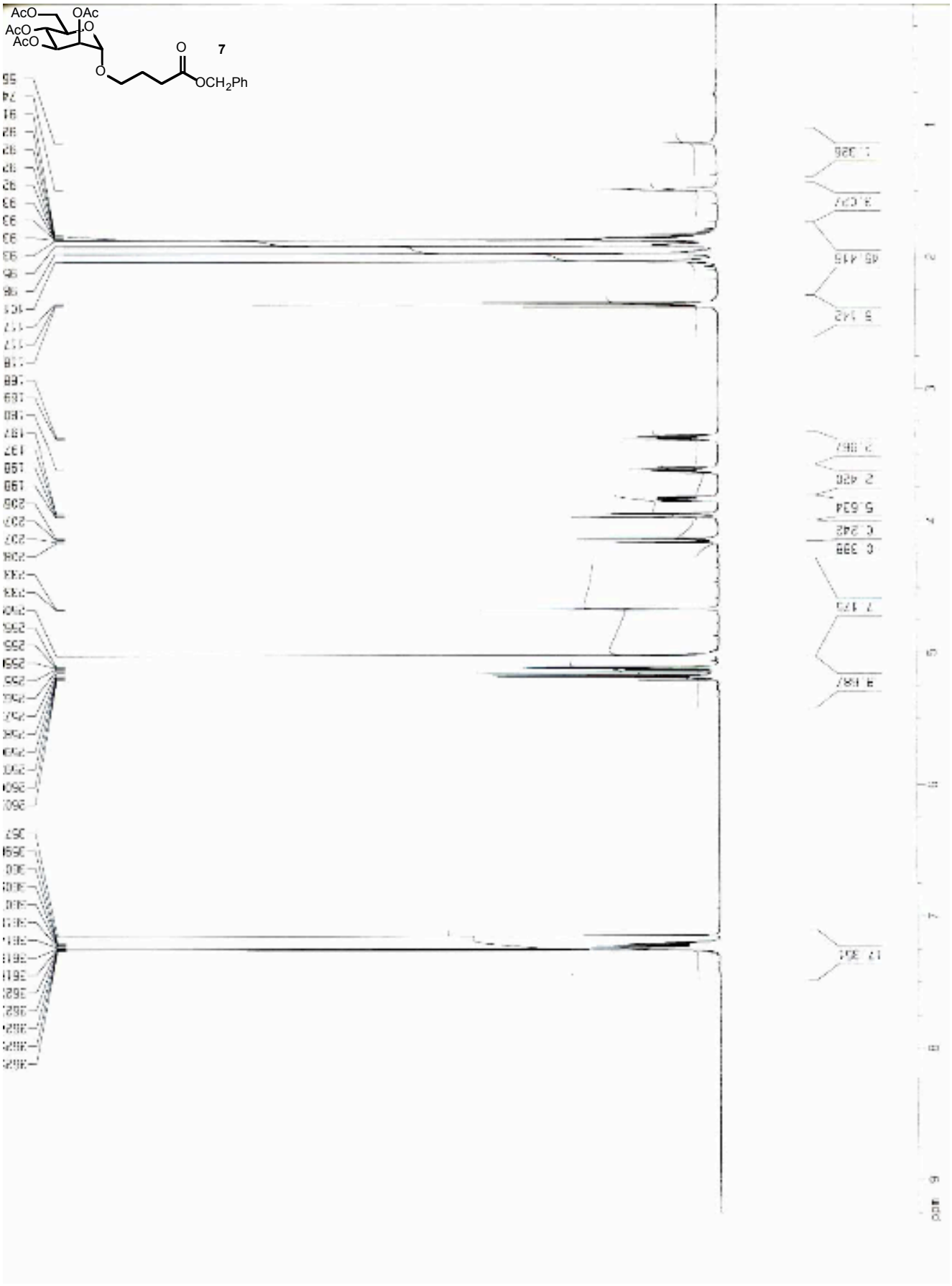
Figure 5. MALDI-TOF MS of **4**

References

[1] M. Dubois, K. A. Gilles, J. K. Hamilton, P. A. Rebers, F. Smith, *Anal. Chem.* **1956**, *28*, 350 - 356.

[2] R. Fields, *Meth. Enzymol.* **1972**, *25*, 464 – 469.

[3] A. C. C. Spadaro, W. Draghetta, S. Nassif del Lama, A. C. M. Camargo, L. J. Greene, *Anal. Biochem.* **1979**, *96*, 317 – 321.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AcO
O
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O
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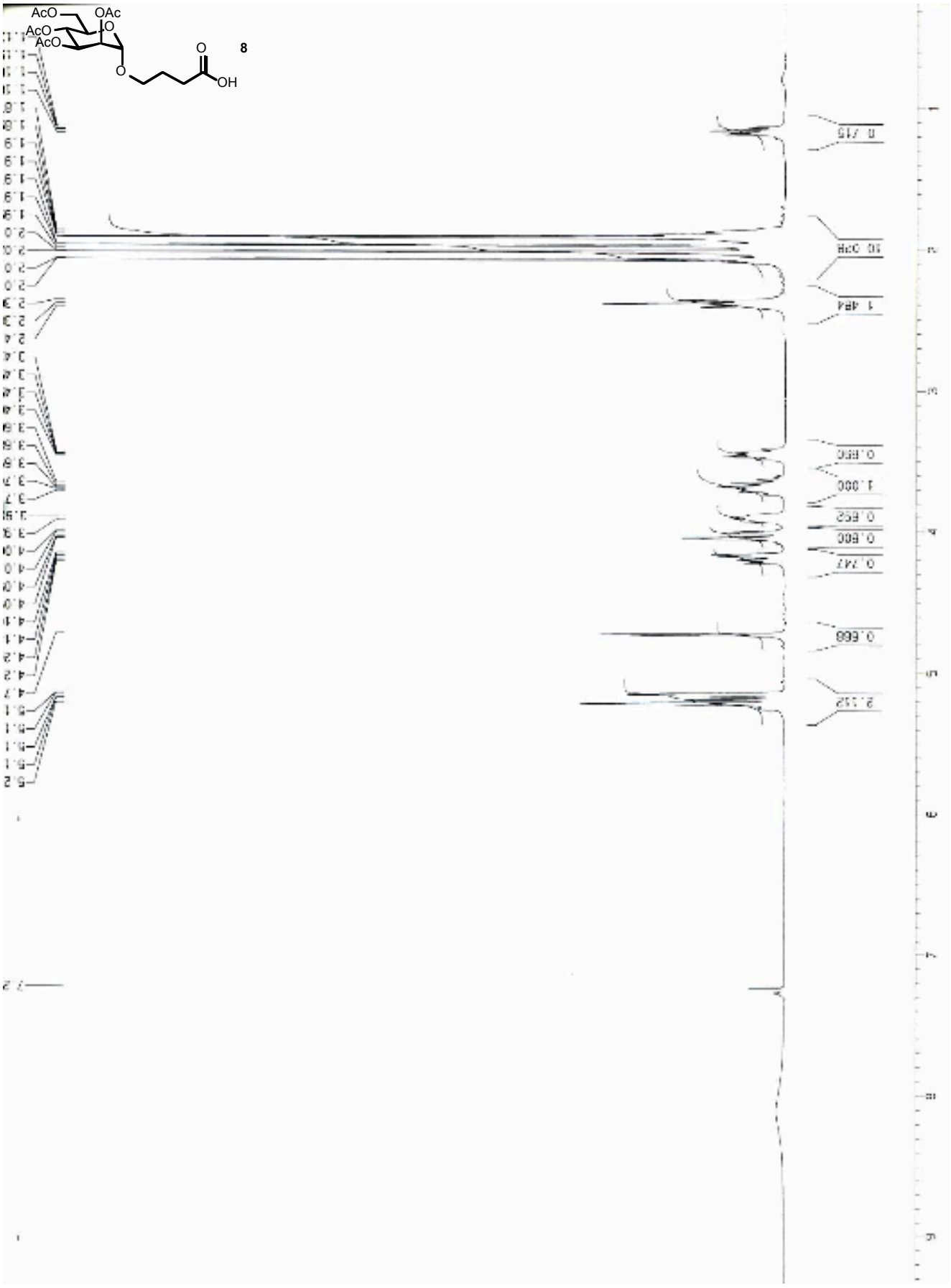
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O
O
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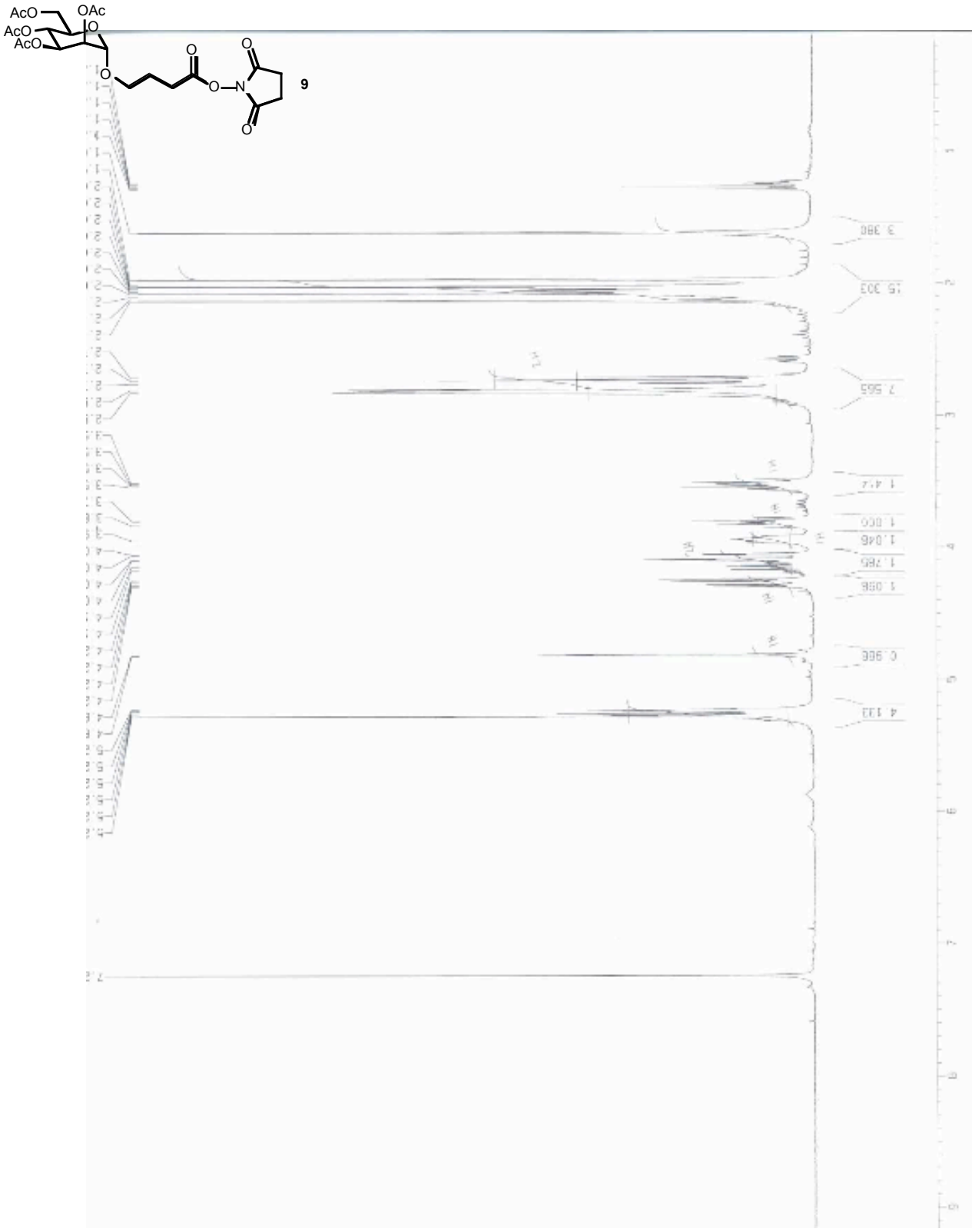
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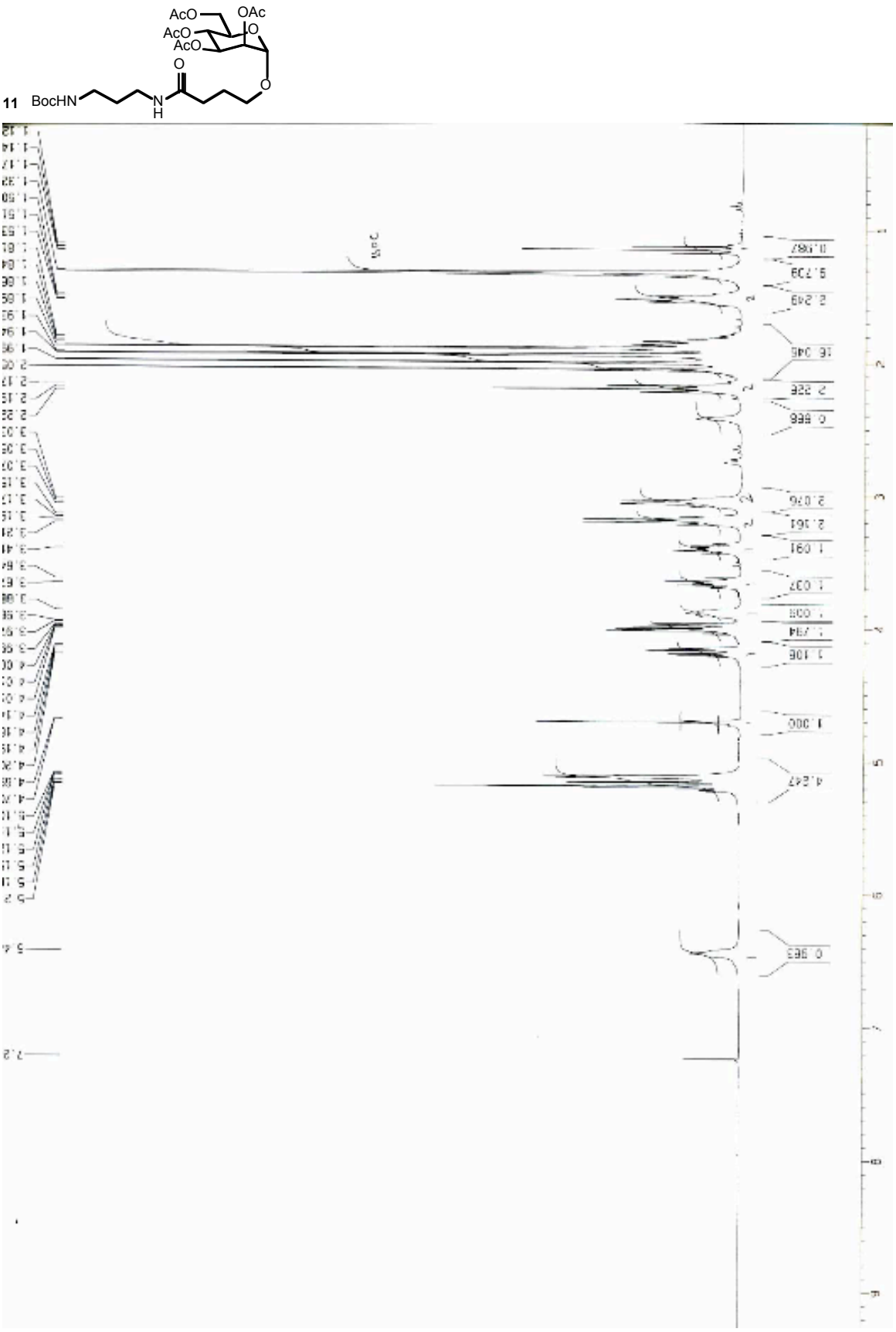

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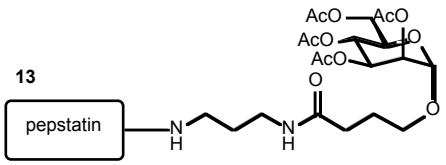

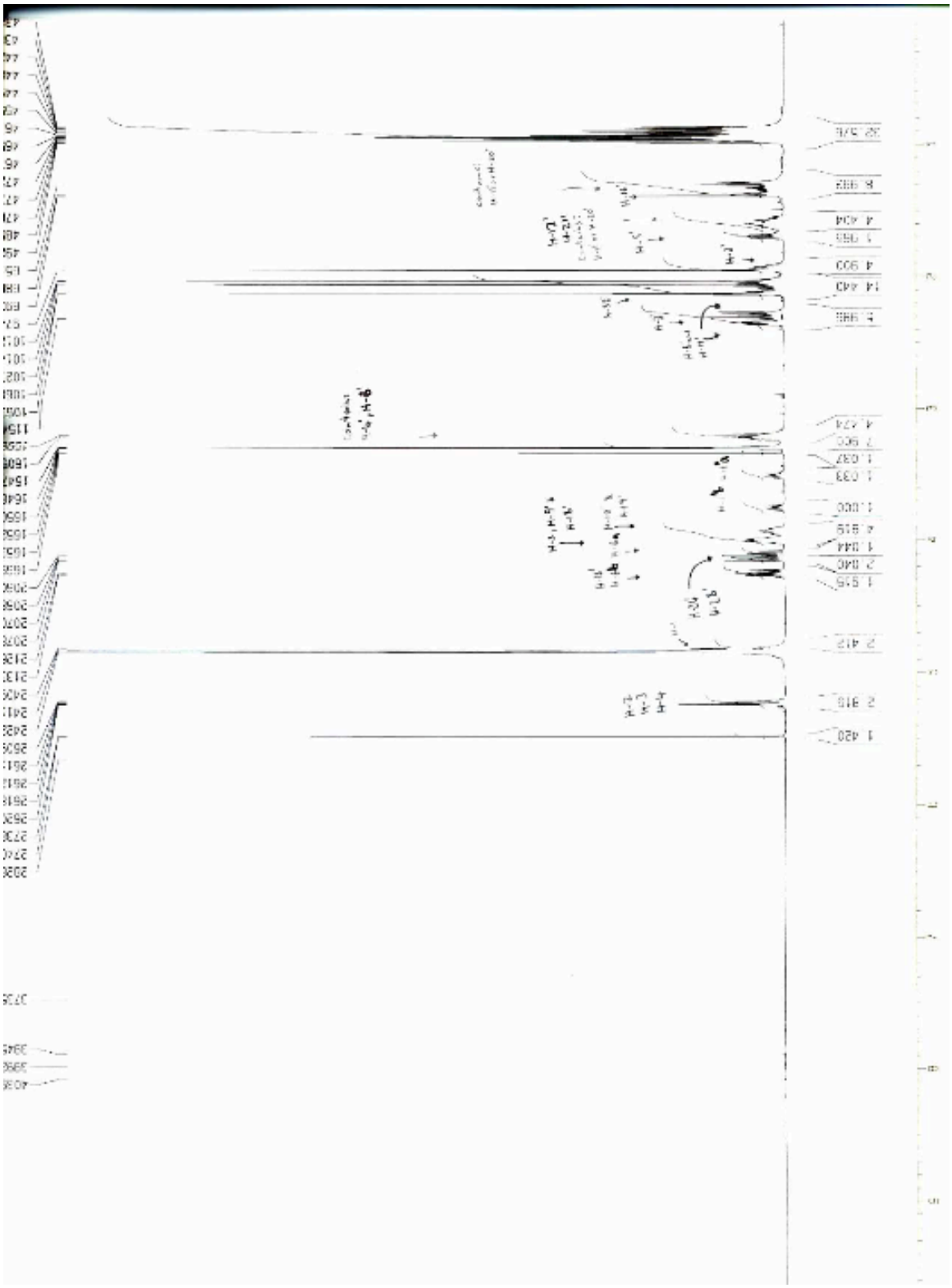

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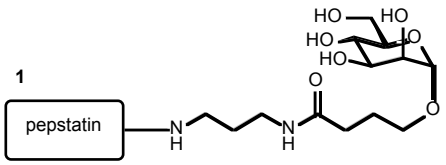

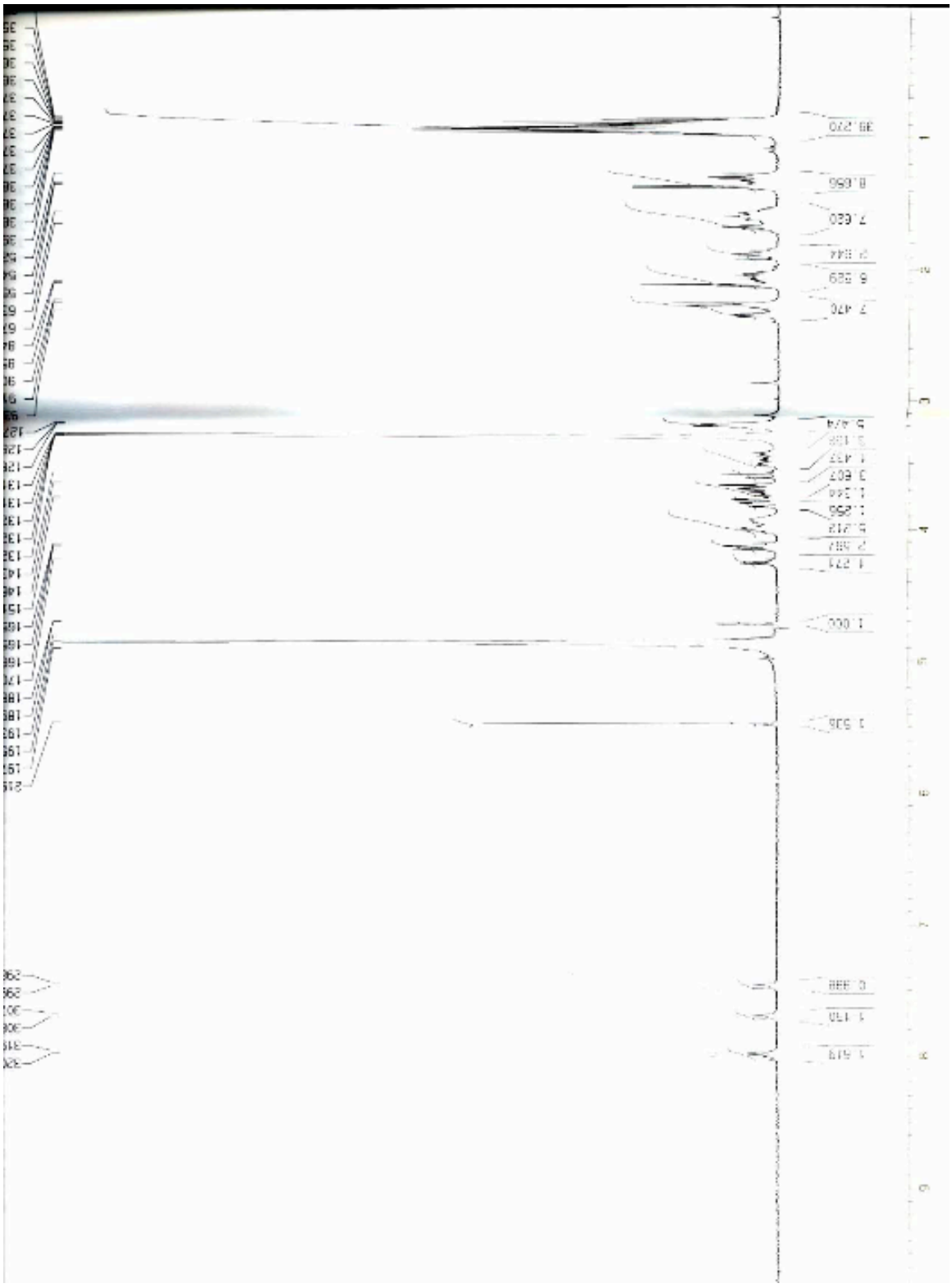

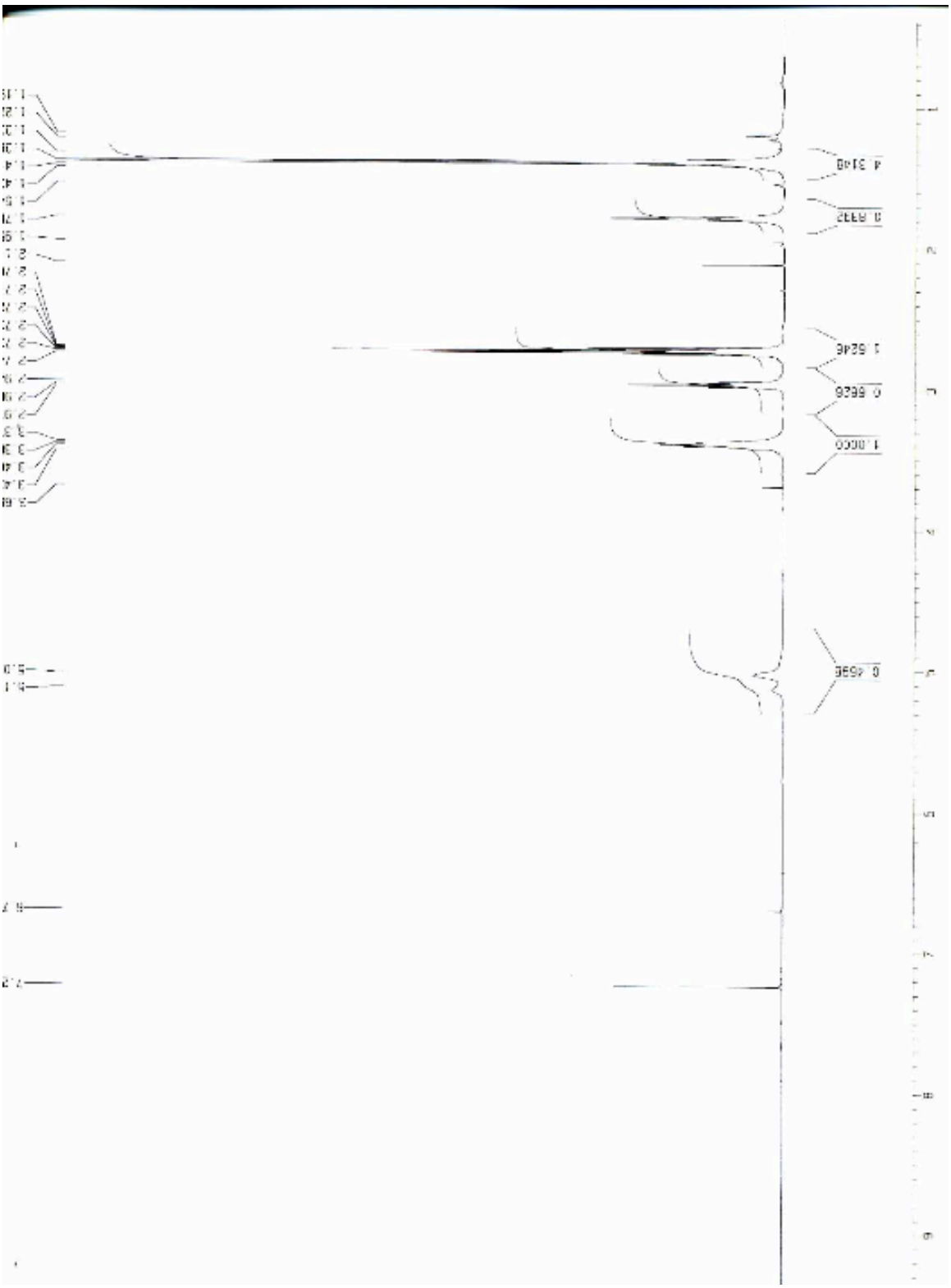

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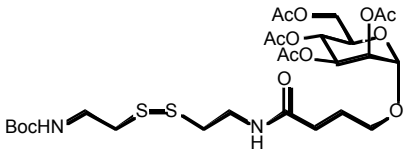

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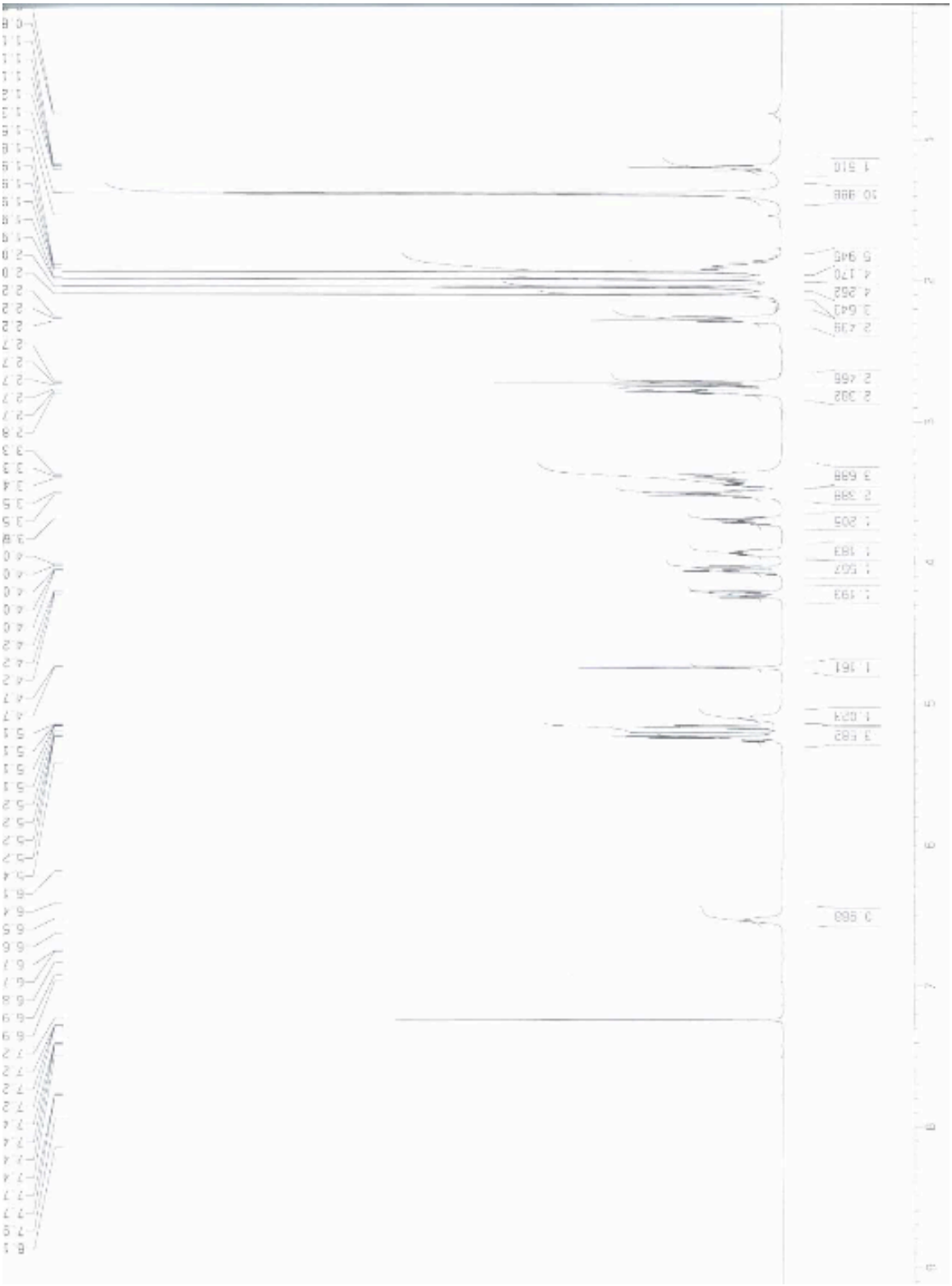

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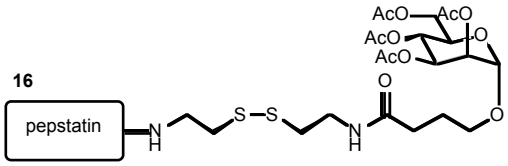

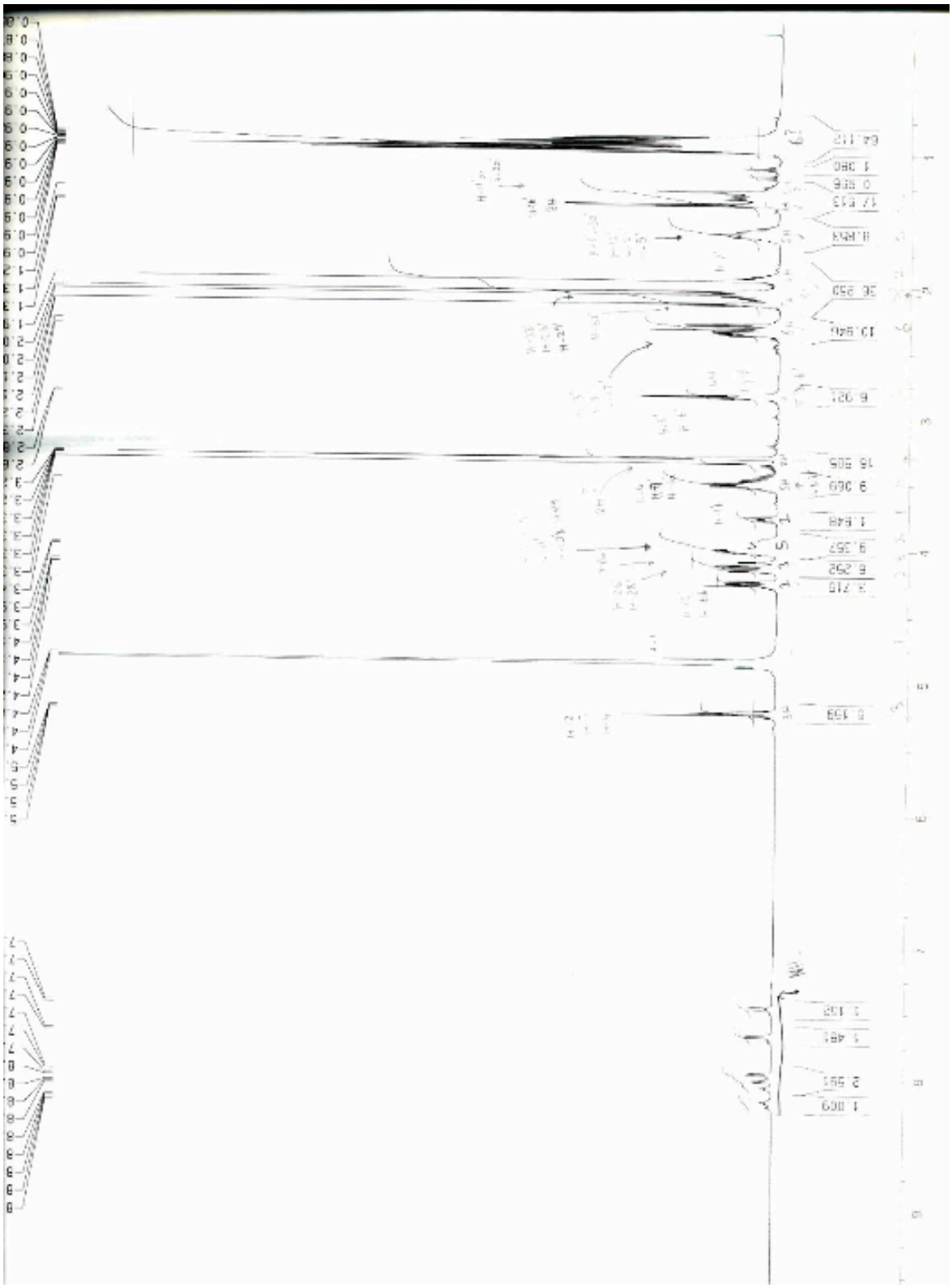

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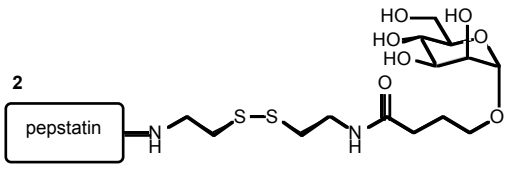
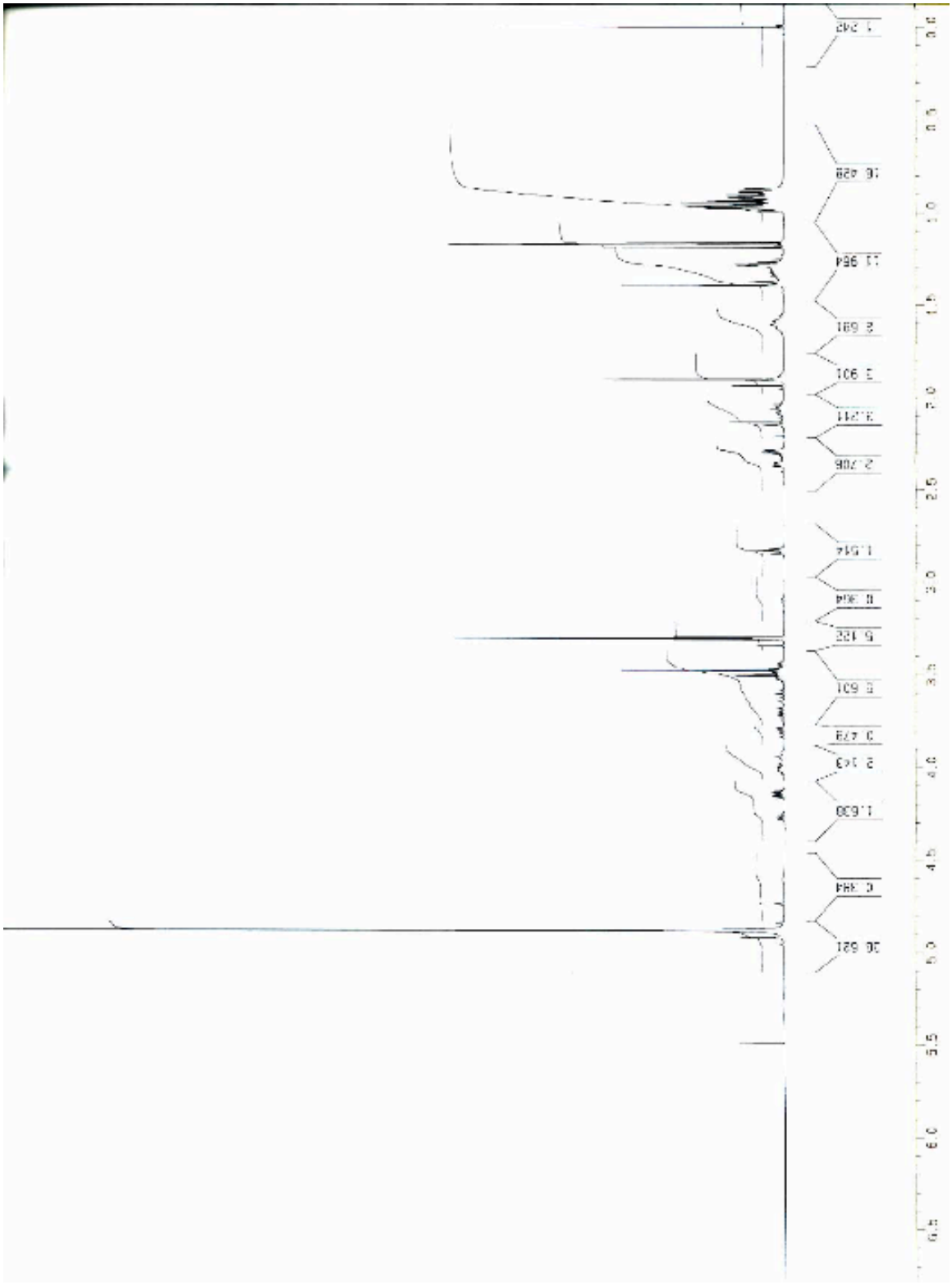
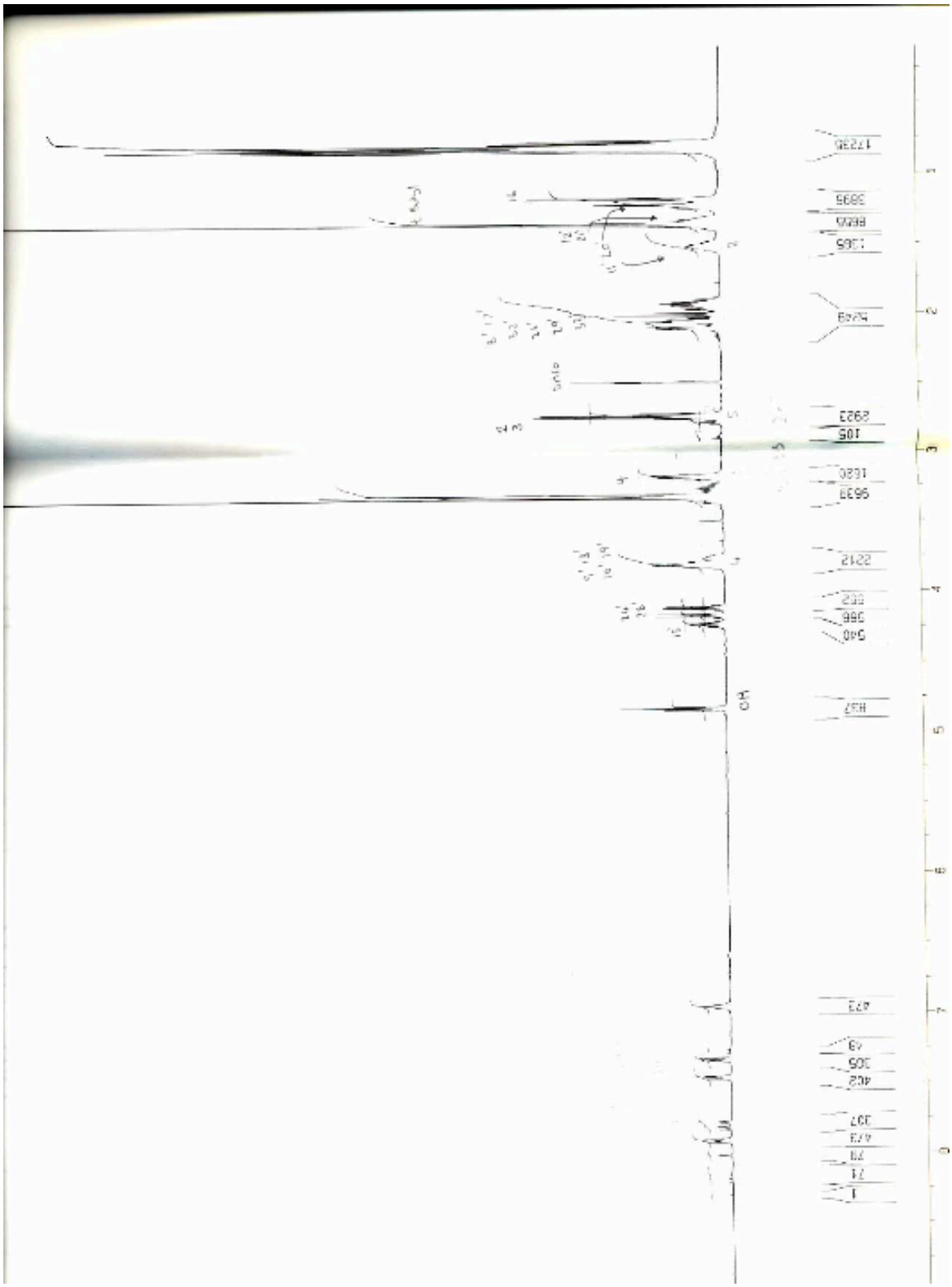
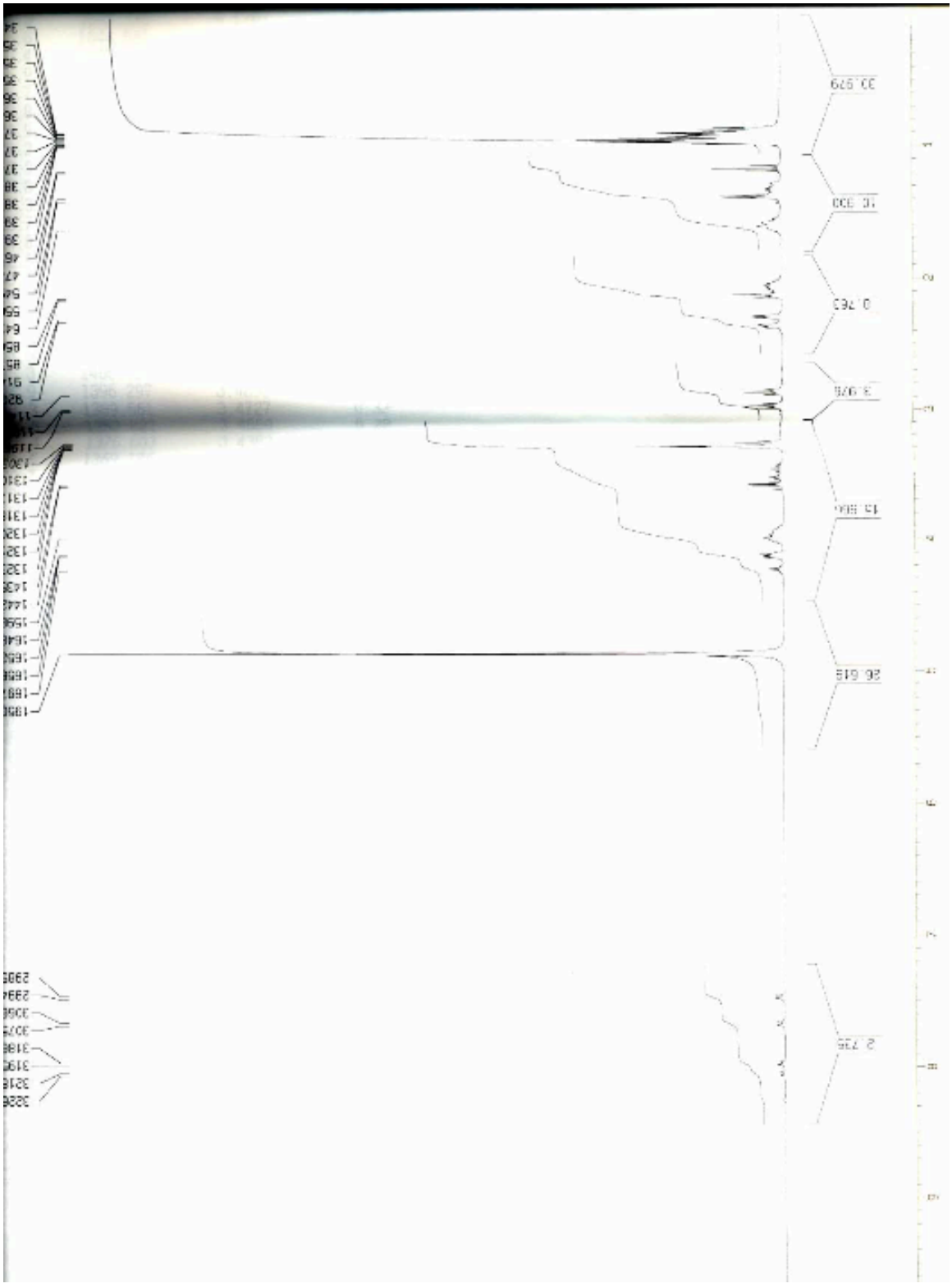


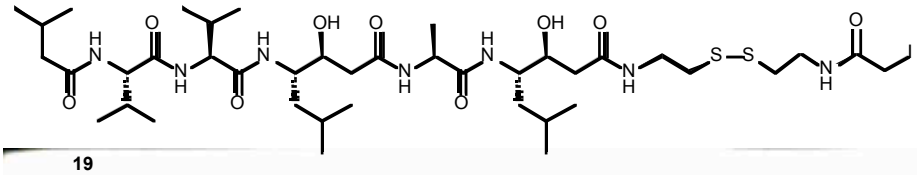


N-(t-Butyloxycarbonyl), N'-(pepstatinyl) cystamine



N-(Pepstatinyl) cystamine





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