Structural Characterization of the aromatic polyketide produced by *S. flaviscleroticus*.

Antibiotics are synthesized by pathways, which are often connected and influenced by primary metabolism; the intermediate metabolites from primary metabolism serve as precursors for biosynthesis of the antibiotic. In fact, the composition of the culture medium, closely connected with the metabolic capacities of the producing organism, greatly influences the biosynthesis of antibiotics. These active molecules are generally extracellular and their isolation in highest purity from the complex fermentation broth needs the application of a combination of various separation steps such as solvent extraction, chemical precipitation, column chromatography, ion exchange chromatography, preparative TLC, HPLC purification, etc. Elucidation of the structure of the compound requires different spectral and mass data like IR, ^{1}H NMR, ^{13}C NMR, COSY, FAB-Mass, LC-MS.

Standardization of production, Isolation and Purification of Polyketide:

For optimal production of the bioactive polyketide (corresponding to that lacking in the mutant, JP1), culture of *S. flaviscleroticus* was streaked on different media like MBA, R_2YE, R_4, SMA. It was observed that in SMA, this compound was produced in optimum amounts. For bulk preparation of the compound, growth from 100 SMA plates incubated for seven to ten days was used and extracted as described in Materials and Methods.

Different solvents like ethyl acetate, chloroform, acetone, methanol, butanol, dichloromethane, hexane and benzene were tested for extraction of polyketide produced by *S. flaviscleroticus*. Ethyl acetate, chloroform, acetone, methanol, butanol, dichloromethane extracts retained both antibacterial and antifungal activities of this organism whereas hexane and benzene extracts did not contain any. Among different solvents tested for extraction of bioactive principle, ethyl acetate was found to be most effective as the inhibition zone on the bioautogram for equal amounts compound loaded from each extracts was largest. In light of this result extraction of 100 plates' culture was

done in Ethyl acetate and concentrated, as described in materials and methods. Purification of the bioactive compound from the extract of *S. flavisclerotícus* (corresponding to that lacking in the mutant extract) was standardized.

The ethyl acetate concentrate was chromatographed on silica column; the impurities removed by washing with chloroform, allowed to separate, and eluted by increasing polarity of $CHCl_3$: MeOH (up to 5%). When methanol concentrations were raised to 5%, the yellow, UV fluorescent, putative polyketide compound started eluting out of the column and continued eluting till the concentration was raised to 7%. When the entire compound from column was eluted, it was concentrated using rotary evaporator.

Further purification of the compound by preparative TLC using 90:10:: $CHCl_3$: MeOH was carried out. The compound was leached out of silica using ethyl acetate + methanol (70:30). Pure compound was monitored on flourophore TLC plate (Fig. 4.1), and in HPLC separation (Fig. 4.2).

Furthermore, the bioactivity associated with the compound was lost when it was leached by methanol from TLC plates prepared in water. However, if the compound is extracted in methanol after TLC separation on silica slurry prepared in 0.5% KH_2PO_4, the bioactivity was found to be highly stable. It was also stable if stored in citrate-phosphate buffer at pH 6.0. The purified sample IIIA (Fig. 4. 1) was checked for its purity by HPLC, for its RT being 34' (Fig. 2) and was analysed by ^{1}H and ^{13}C NMR at Sun Pharma, Vadodara; 2D COSY at TIFR, Mumbai, and LC-MC at IICT, Hyderabad. In the lab it was analyzed using the following techniques.

Spectroscopic Analysis:

The purified compound was subjected to spectroscopic analysis: two absorbance maxima, one at 285nm (suggested aromaticity) and other at 420nm (due to yellow chromophore) was observed. The bioactive compound also contained an active fluorophore - when excited at 410nm and 435nm, fluorescence maxima at 490nm and 520nm respectively, were obtained (Fig. 4.3).

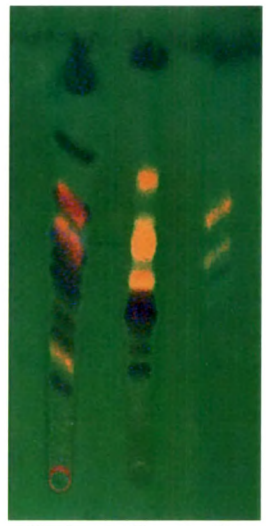


Fig.4.1: Testing by TLC the purity of the compound: Crd- crude extract of *S. flavisclerotícus* culture, C- Column purified compound, P- pure compound.

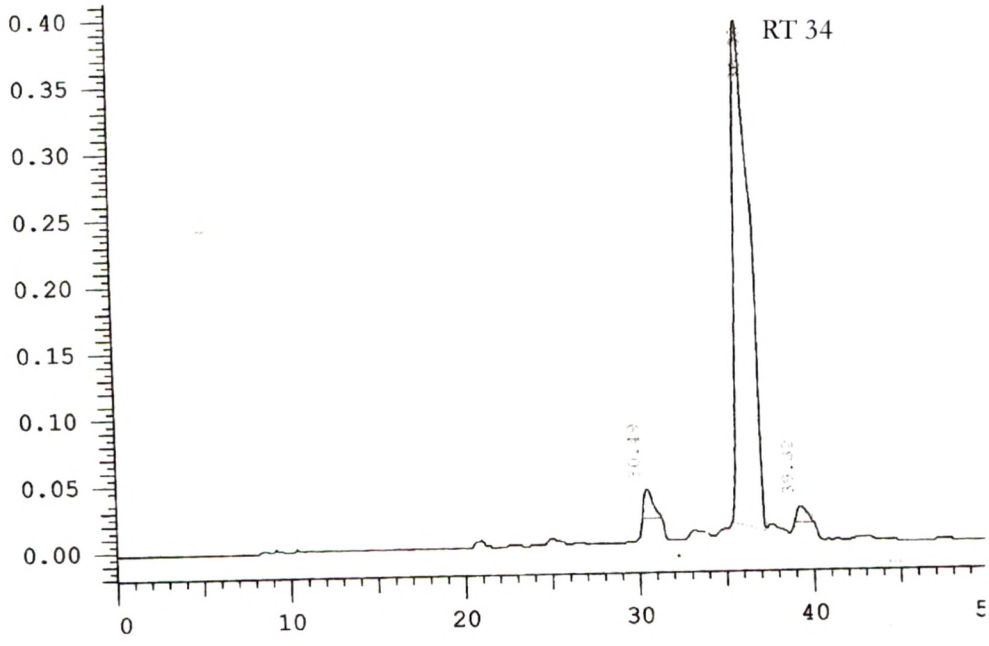
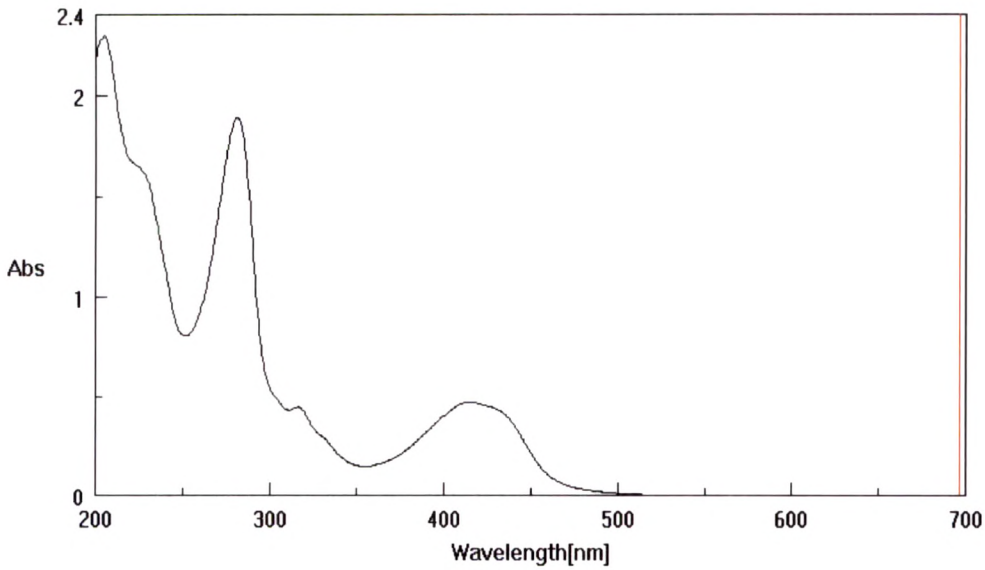


Fig. 4.2: HPLC Chromatogram of purified compound IIIA.



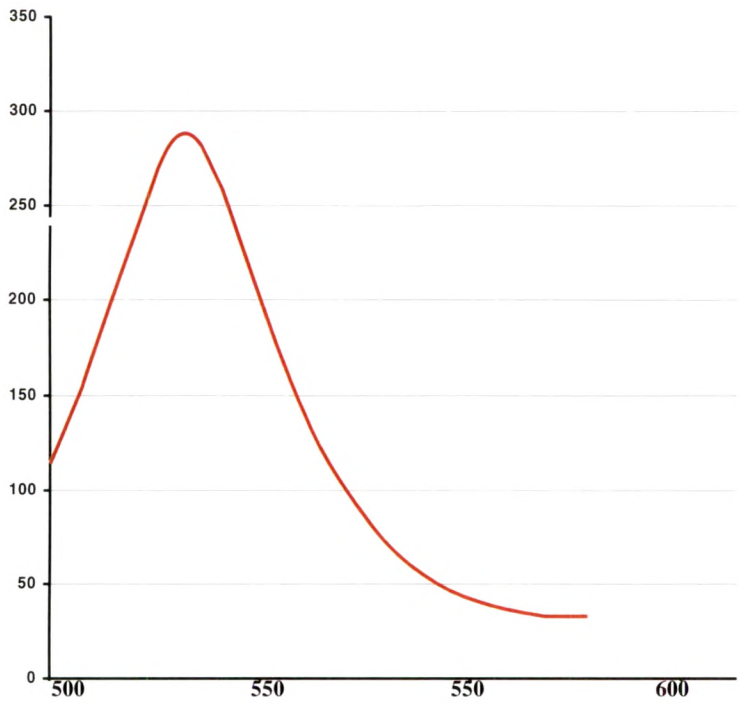


Fig. 4.3: A) UV absorption and B) Fluorescence spectra of pure fraction

TLC separation and bioautogram

TLC separation and bioautogram

The compound so obtained has two closely moving compounds, II and III on 1D TLC (Fig.4.4); when separated by 2D TLC, the compounds are further resolved into three components II, IIIA and IIIB, they are fluorescent orange under UV light (Fig. 4.5). *B subtilis* or *M. luteus* growth is inhibited by these three spots as may be seen in the bioautogram (Fig. 4. 5) developed using either of them as test organism.

HPLC separation, TLC and agar well assay of fractions: Purified compound which contained the three fractions II, IIIA and IIIB were separated by HPLC according to the program No.2, described in materials and methods. The fractions at RT, 28', 32' and 34' were collected. The fractions were pooled, concentrated and separated on TLC and compared with crude extract of the organism of study (Fig. 4.6) Agar well assay of each showed the extract to be bioactive. The fraction at 32' and 34' were sent for LC-MS.

LC-MS: LC-MS of the compounds at RT 32' and 34' was done according to the program No.2 of HPLC. The molecular mass of sodium and potassium adduct of compound at RT 32' were 1206- and 1222 daltons respectively, and molecular mass of sodium and potassium adduct of compound at RT 34' were 1234 and 1250 daltons respectively. Thus, the molecular mass of compound at RT 32' was 1183 daltons and that of RT at 34' was 1211 daltons. Chromatogram and data is attached with the thesis.

The database of chemical compounds indicated that these compound could be chromomycin A_3 (RT-32') and chromomycin A_2 (RT-34'). Also the producer organism, *S. flavisclerituus* of these compound produces all the three forms namely A_2, A_3, and A_4, the third compound by these criteria should be A_4. The pure compound at RT 34' and molecular weight 1121 daltons, which was produced in major amount, was sent for **^{13}C NMR, ^{1}H NMR, 2D COSY,** and **IR.** Also authentic compound A_3 was ordered from

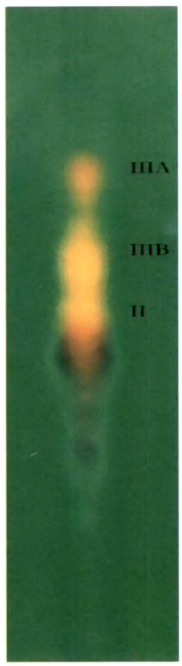


Fig. 4.4: TLC showing closely moving spots II and III.

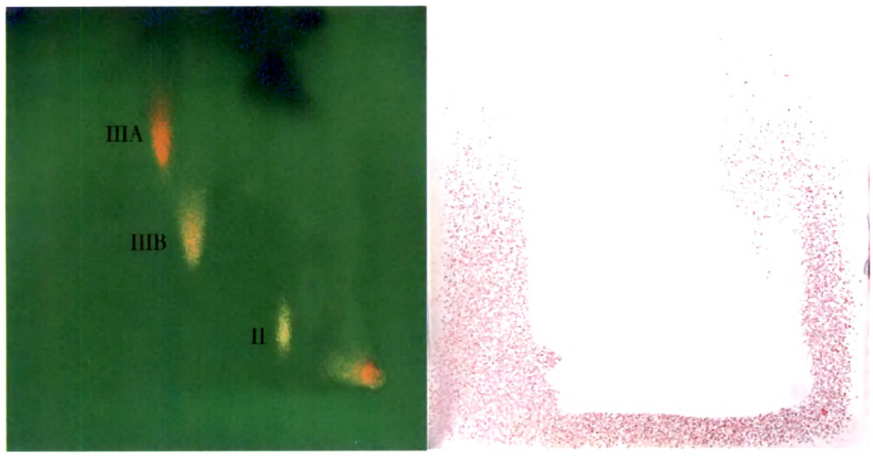


Fig. 4.5: TLC and Bioautogram of purified compounds. A, 2D TLC of purified compound to show three bioactive fractions, II, IIIA, and IIIB; B, Bioautogram using *M. leuteus*.

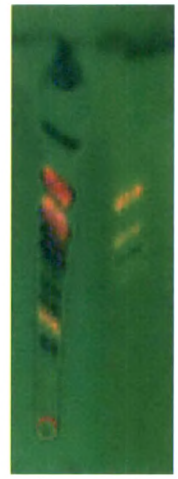


Fig. 4.6: TLC of HPLC fraction, collected and pooled to compare with crude extract of S. flaviscleroticus: 1, crude extract; 2, HPLC fractions pooled and concentrated.

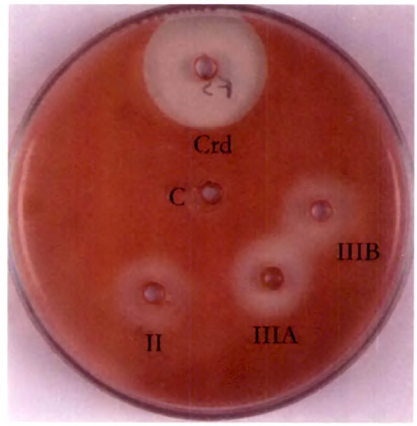


Fig. 4.7: Agar well assay to show bioactivity of fraction collected from HPLC. Crd- crude extract of S. flaviscleroticus, II- fraction from RT 28', IIIA- fraction from RT 32', III- Fraction from RT 34'.

Sigma. HPLC profile of the purified compound prepared in our lab and that obtained from Sigma was carried according to program No. 2 of gradient HPLC.

HPLC of Chromomycin obtained from Sigma and prepared in the lab: The compound obtained from sigma, chromomycin A_2, also had a retention time of 34' when separated using the gradient program No. 2. This further indicated the compound produced by the organism of our study, *S flavisclerotícus,* having RT 34' under the same program of HPLC to be chromomycin A_2 Fig. 4.7.

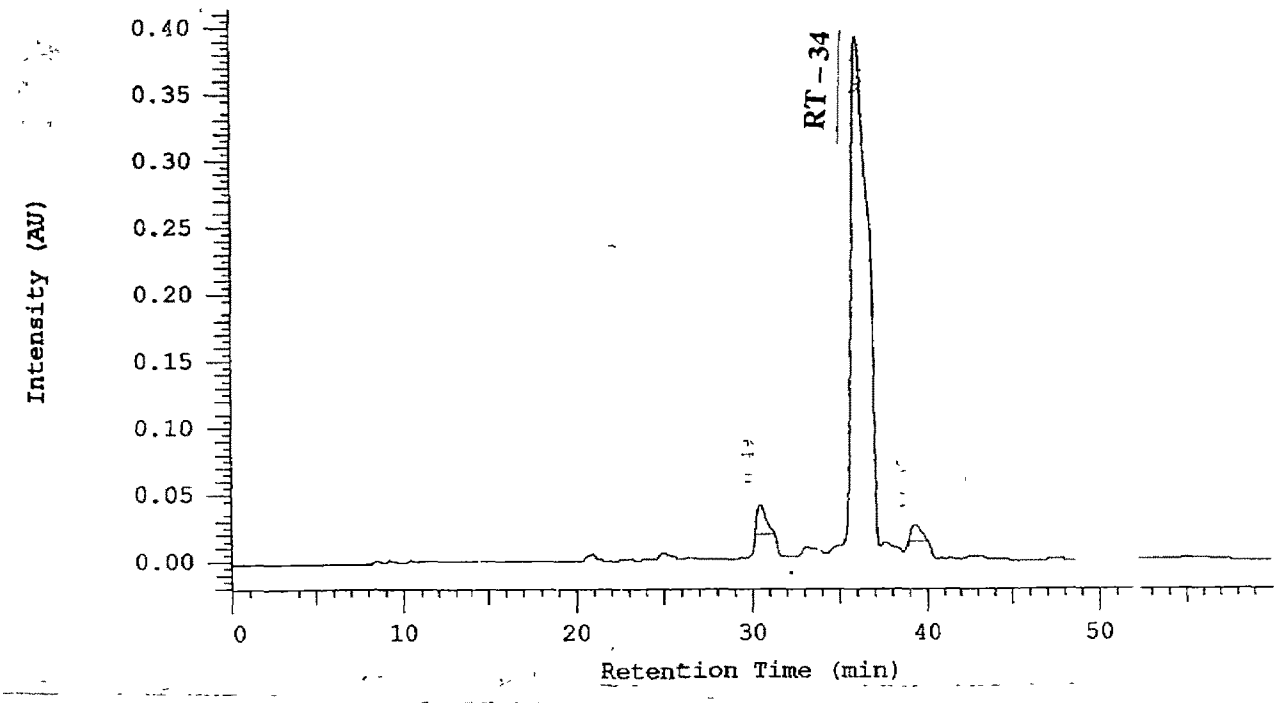
^{13}C NMR, ^{1}H NMR, 2D COSY, and **IR of the compound (III) at retention time 34':** ^{13}C NMR and ^{1}H NMR had aromatic signals at δ (159.5, 156.2, 165.4) and δ (6.63, 6.75) respectively, which is same as that of reported values of chromomycin A_2. Rests of the ^{13}C NMR and ^{1}H NMR signals are also same as that reported of chromomycin A_2, (confirmed by expert opinion).

2D COSY and IR spectra are also same as that of reported spectra of chromomycin A_2 and was again confirmed by expert opinion (Dr Nagaraj, Scientist, CCMB, Hyderabad).

The pure compound was also sent to Dr Jurgon Rohr's lab at University of Kentucky for analysis by NMR and was confirmed to be chromomycin A_2.

Discussion and Conclusions

The antibacterial compound produced by PKS cluster of *S. flavisclerotícus* is chromomycin. Like the other producer of chromomycin, *S. griseus* it produces all the three forms of chromomycin, A_4, A_3 and A_2 which our lab had designated as II, IIIB and IIIA respectively.



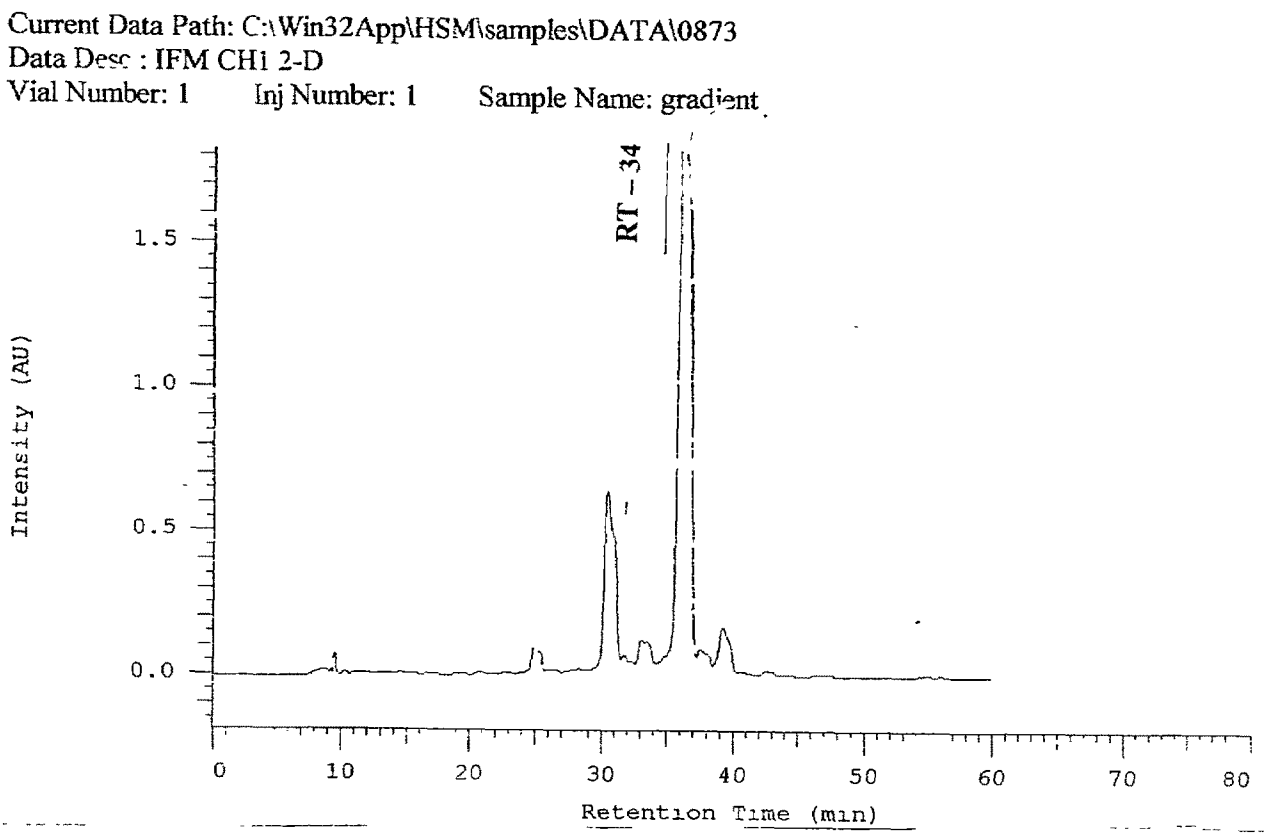
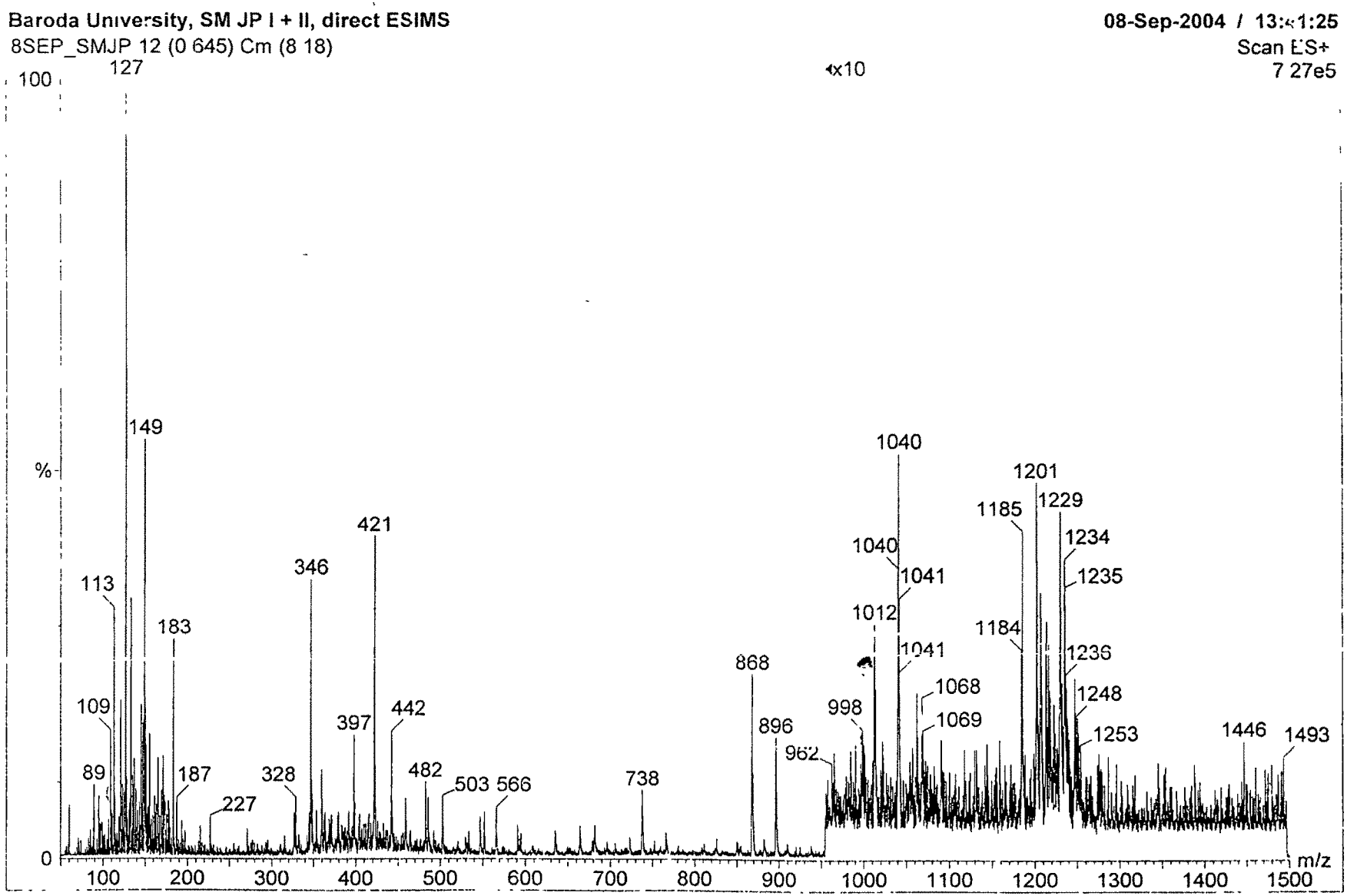
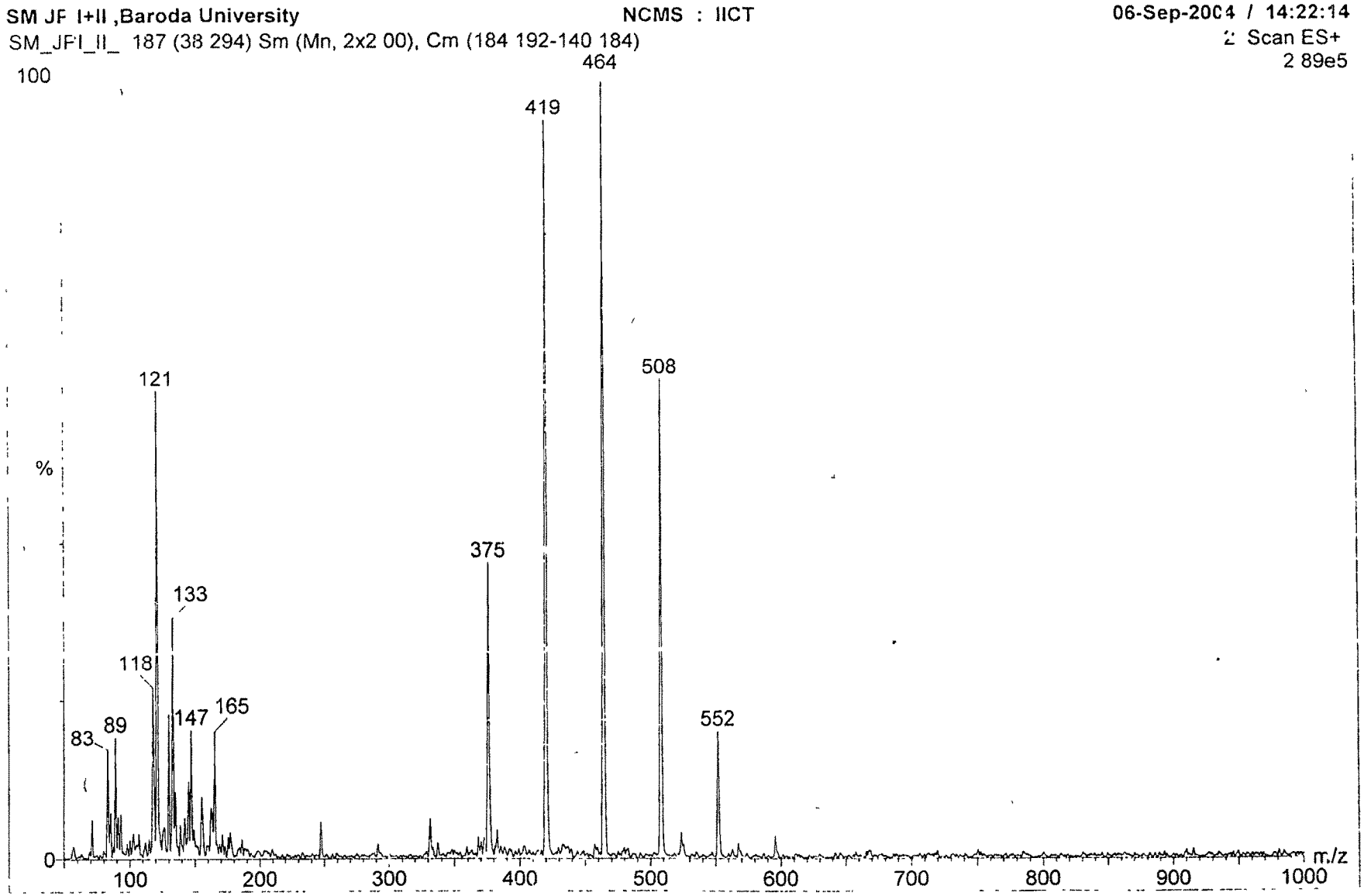


Fig.4.8: HPLC chromatogram of a) Fraction IIIA; b) Chromomycin obtained from Sigma

LC MS 08 Sep
JP I + II



LC-MS of IIIA.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121
375
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118
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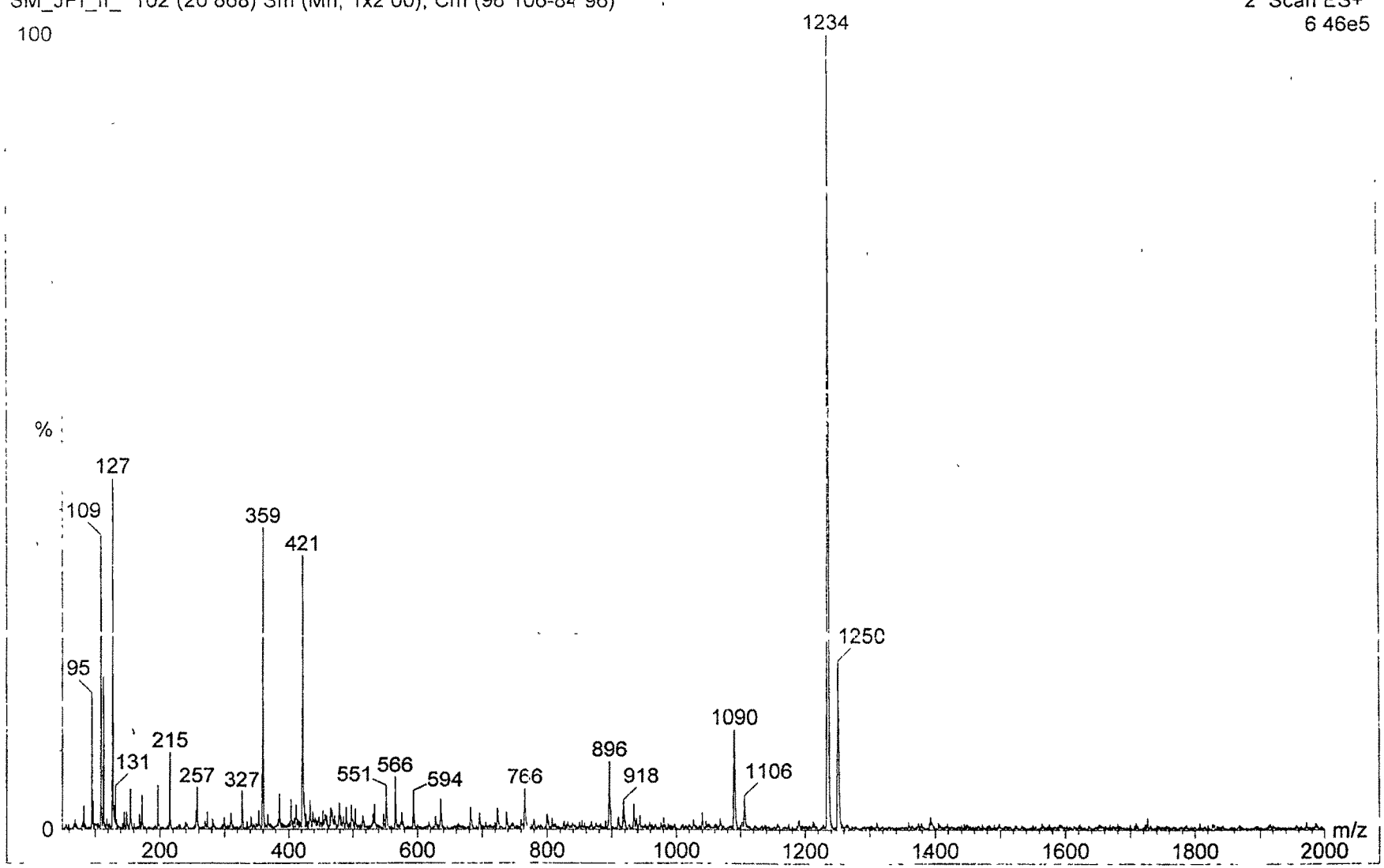

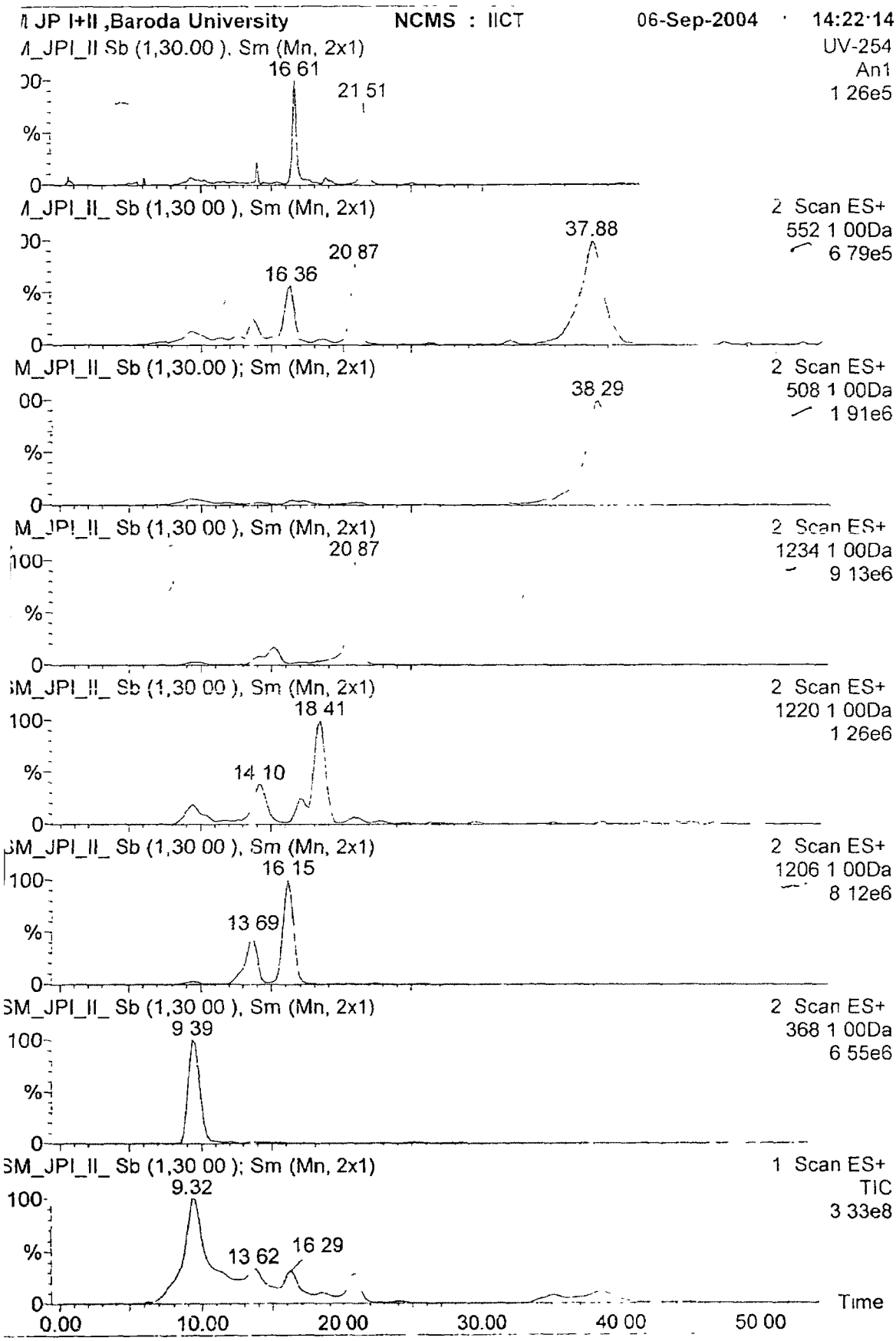
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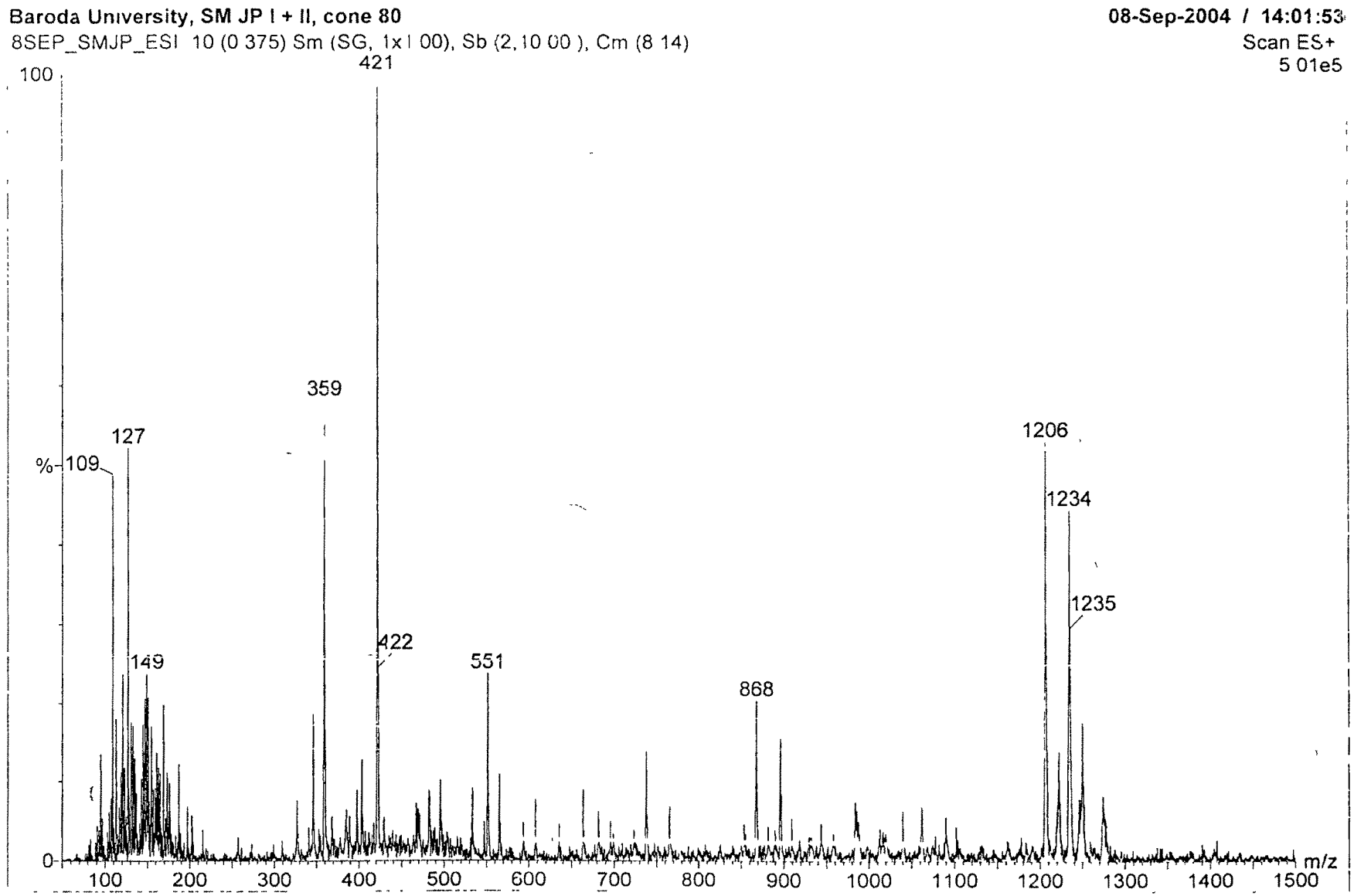
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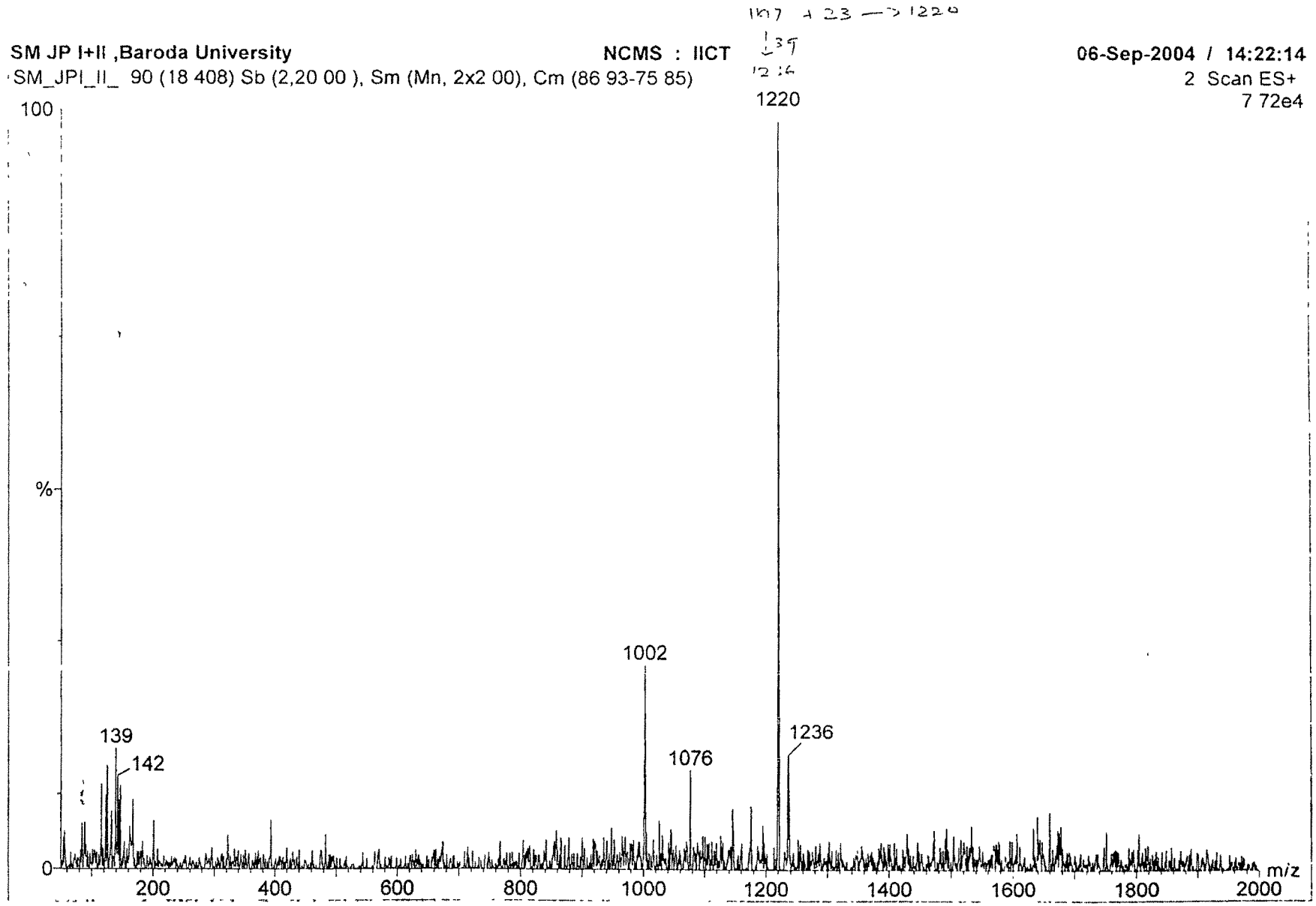
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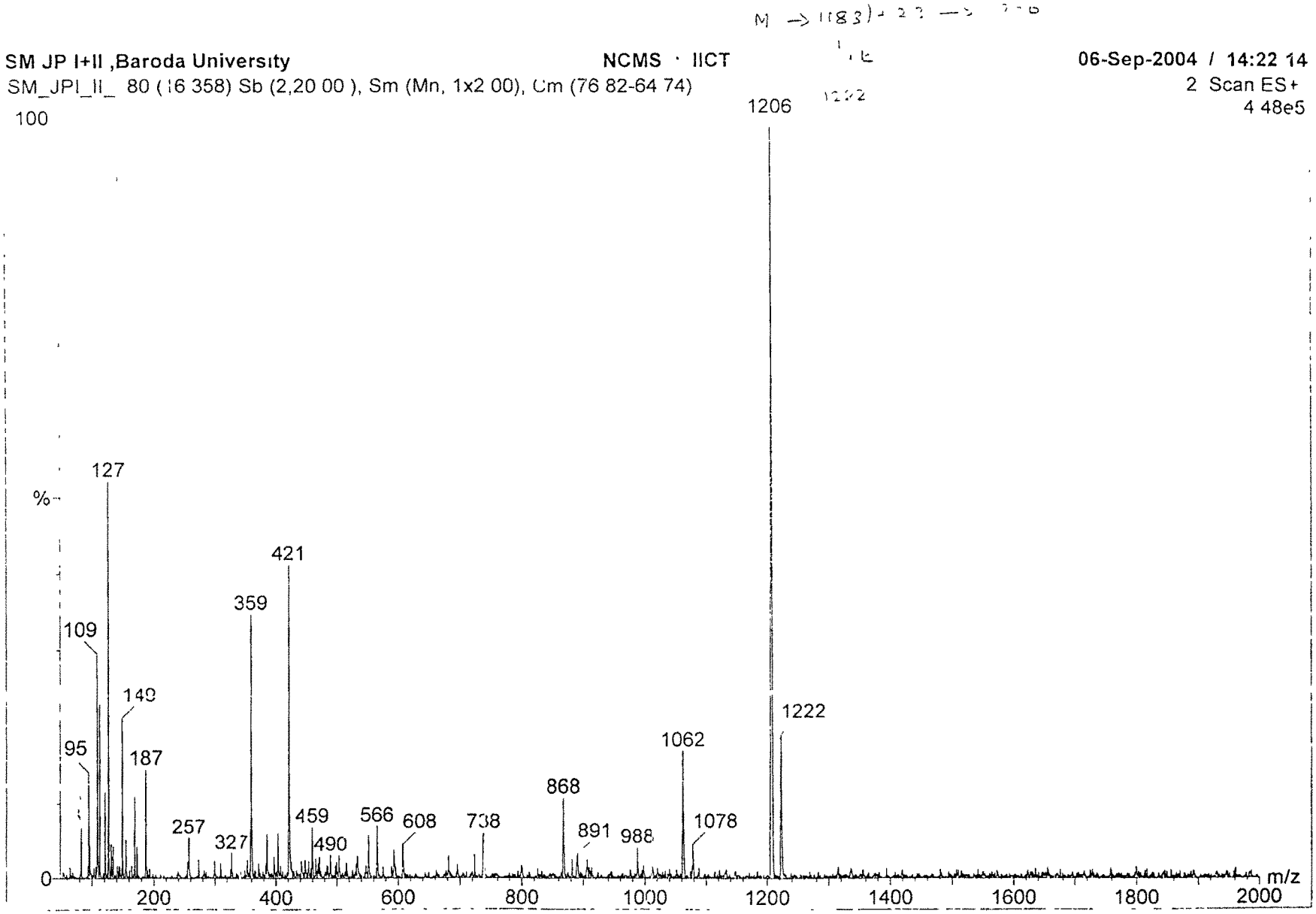
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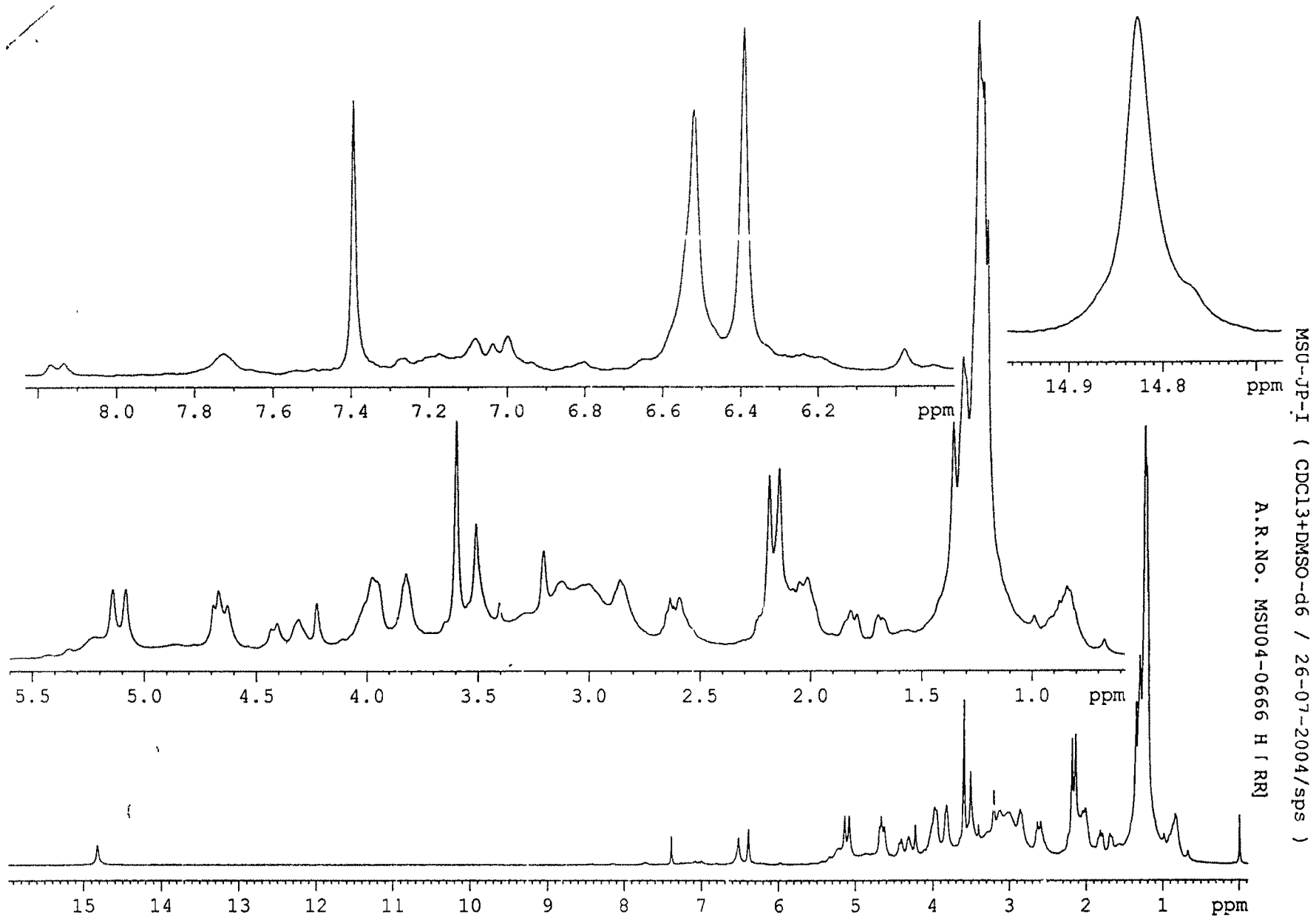
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22	11659.4	1725.625	4.3127	1.62	*
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25	12102 9	1531.172	3.8267	3.40	***
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28	12485.0	1363.649	3.4080	2.28	**
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49	14492.1	483.574	1.2085	17.25	**********
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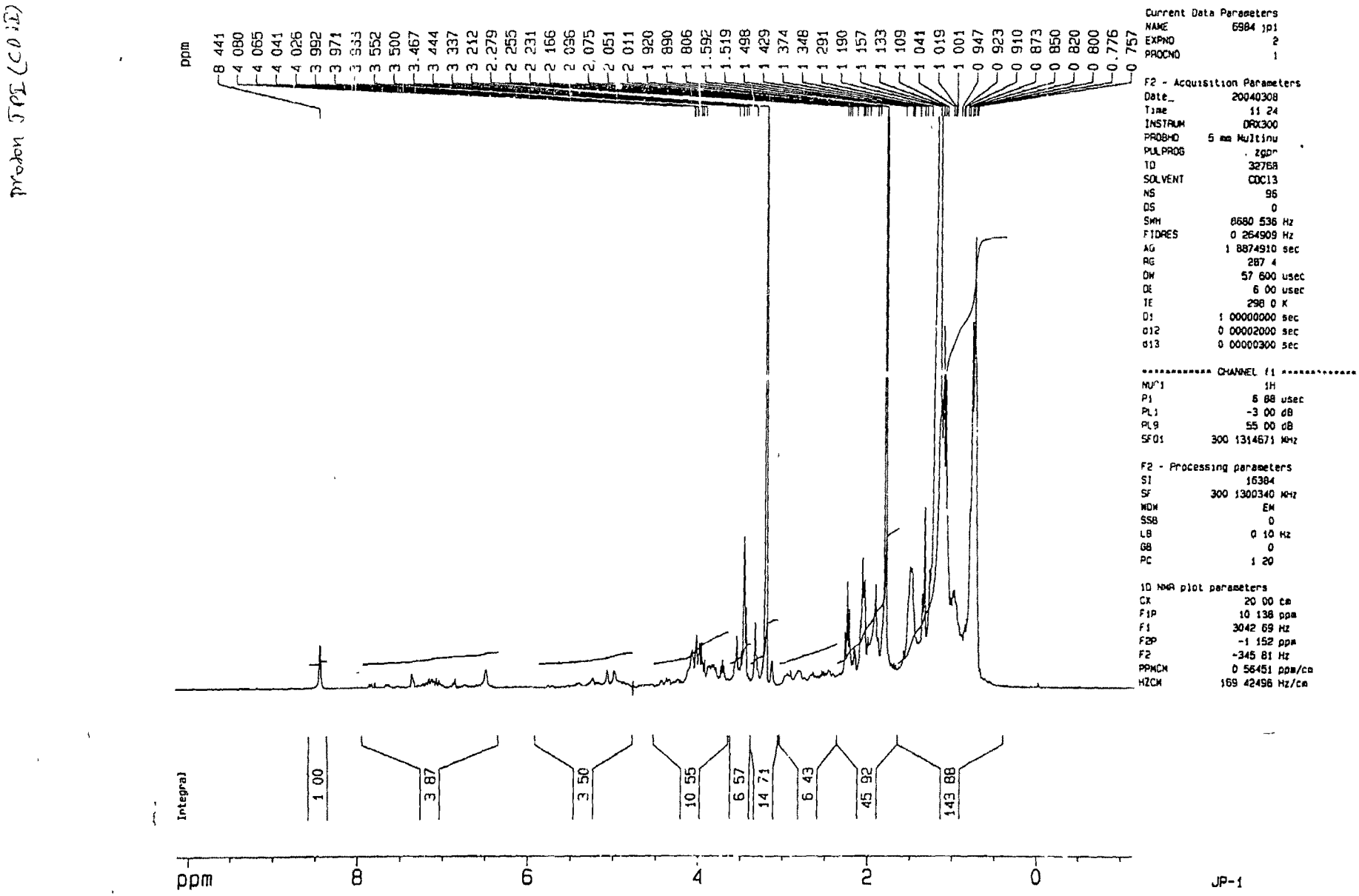
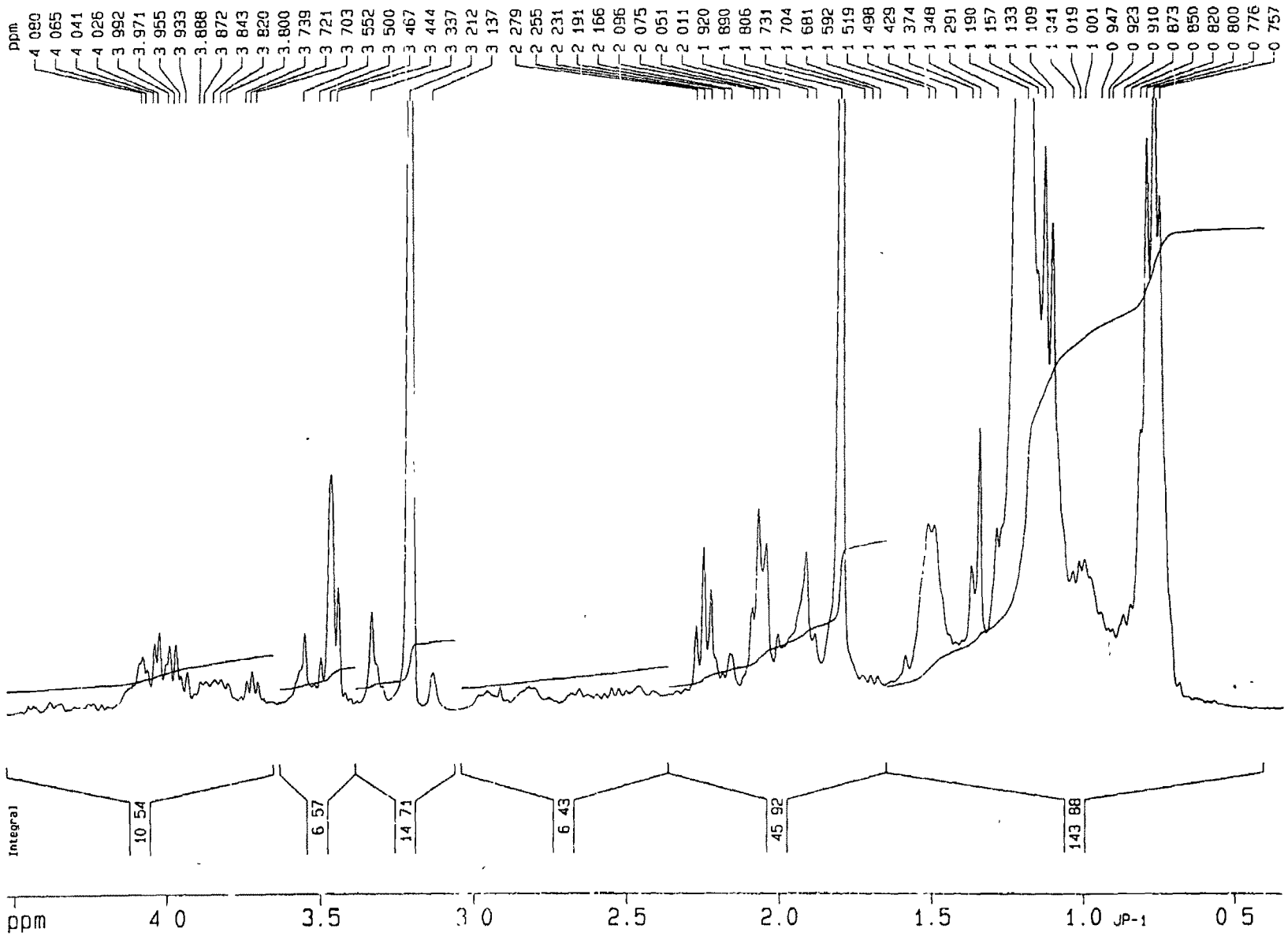


^{1}H NMR data of IIIA.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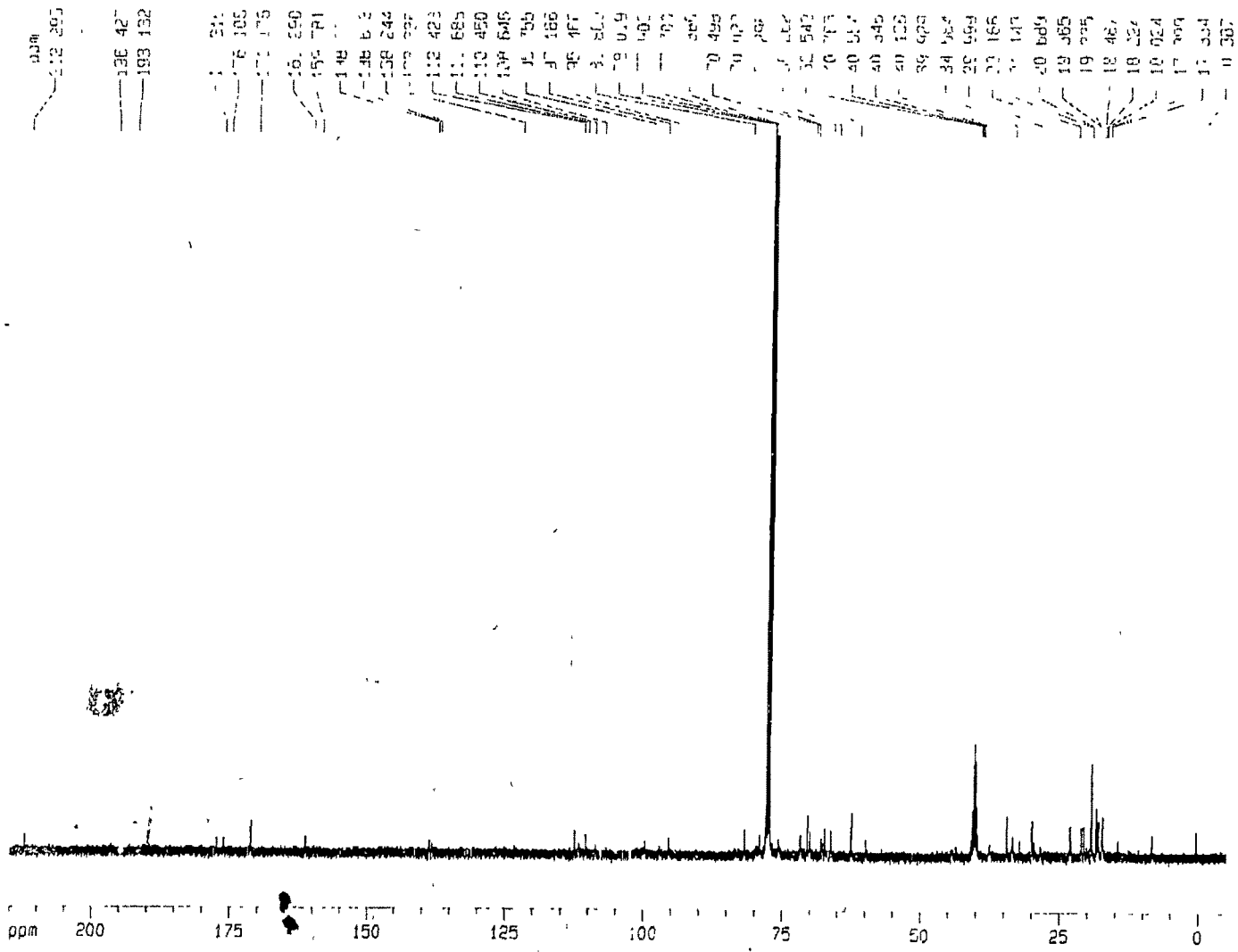

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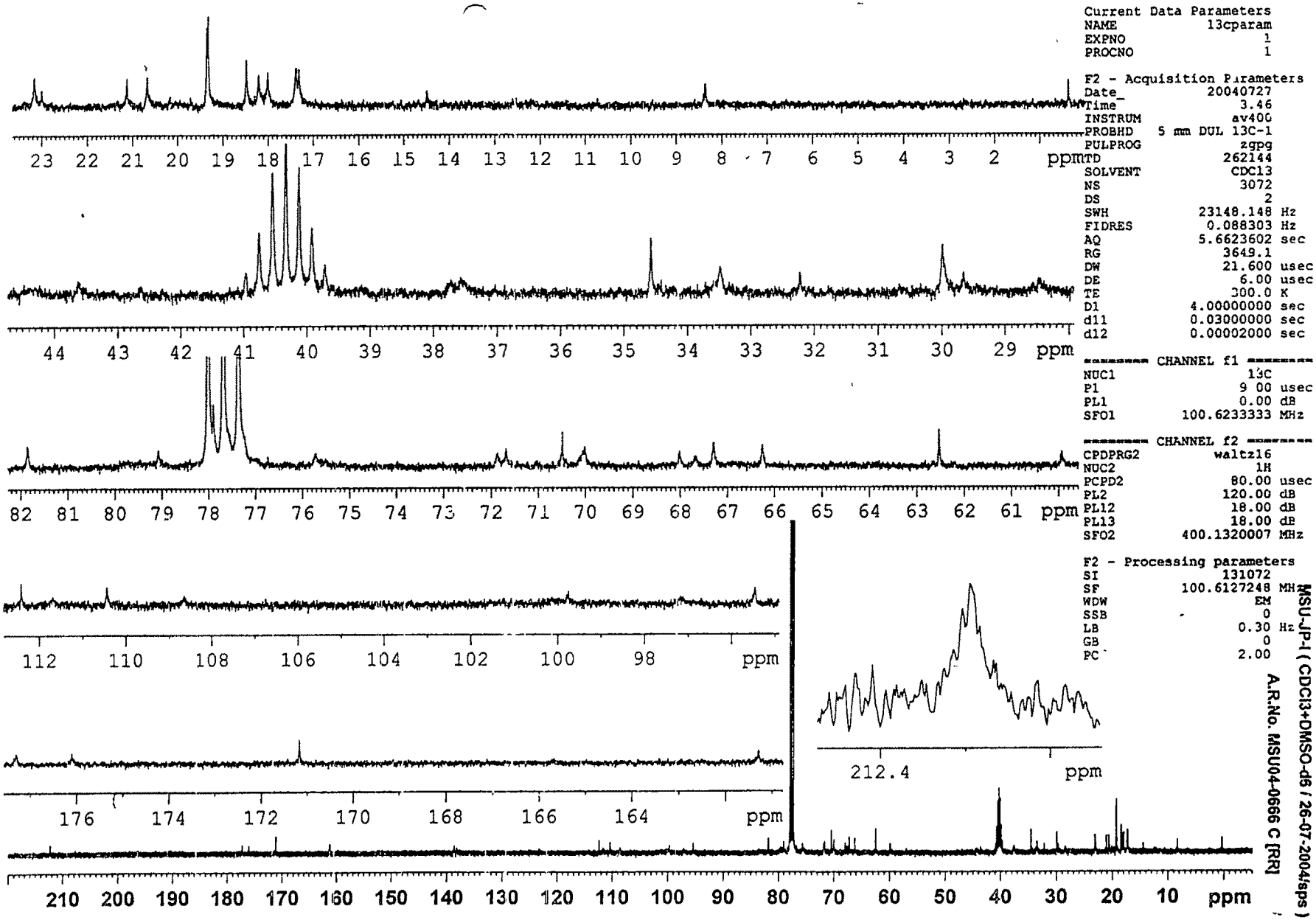
(MS) C13 JPL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PL13 18.00 dB
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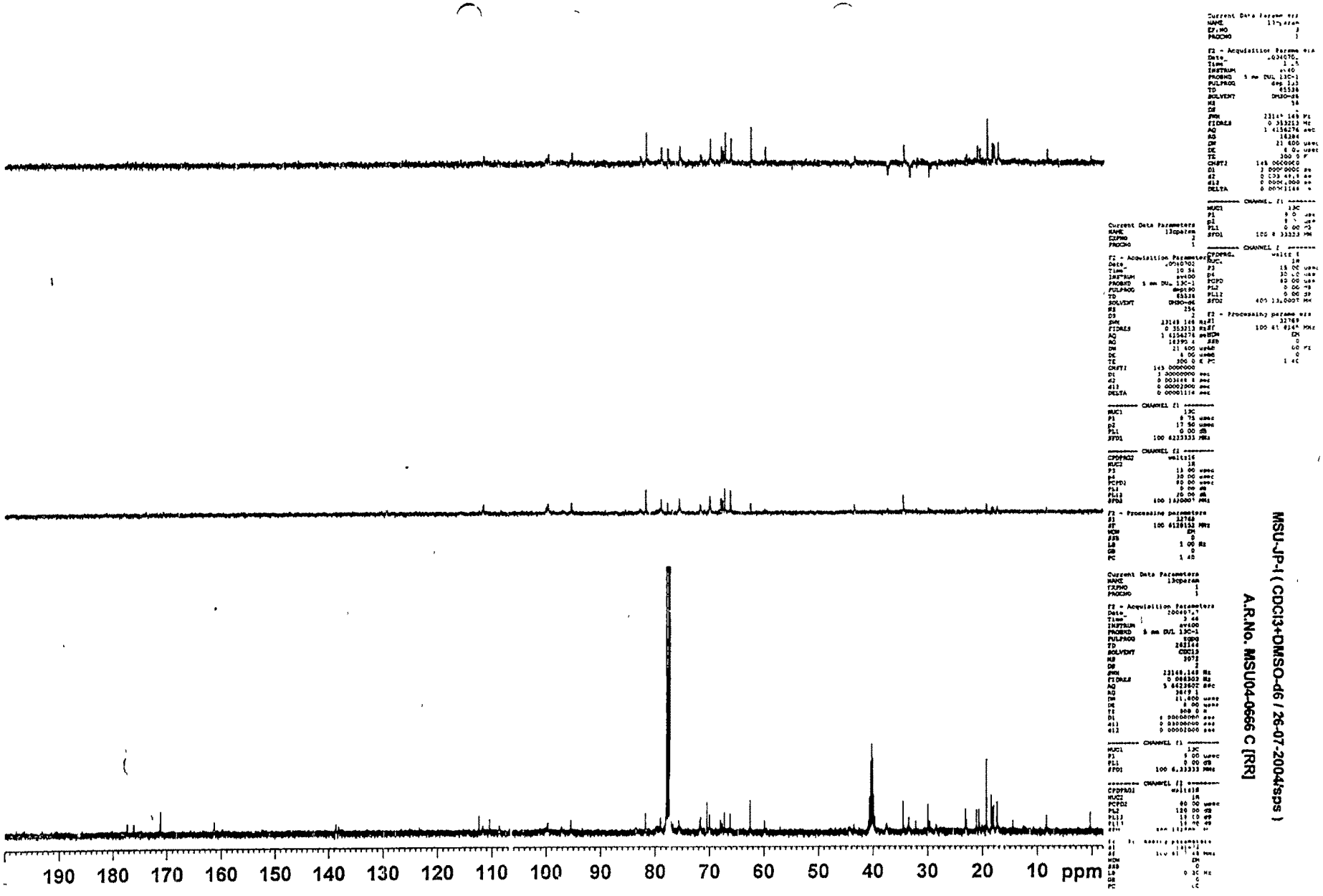

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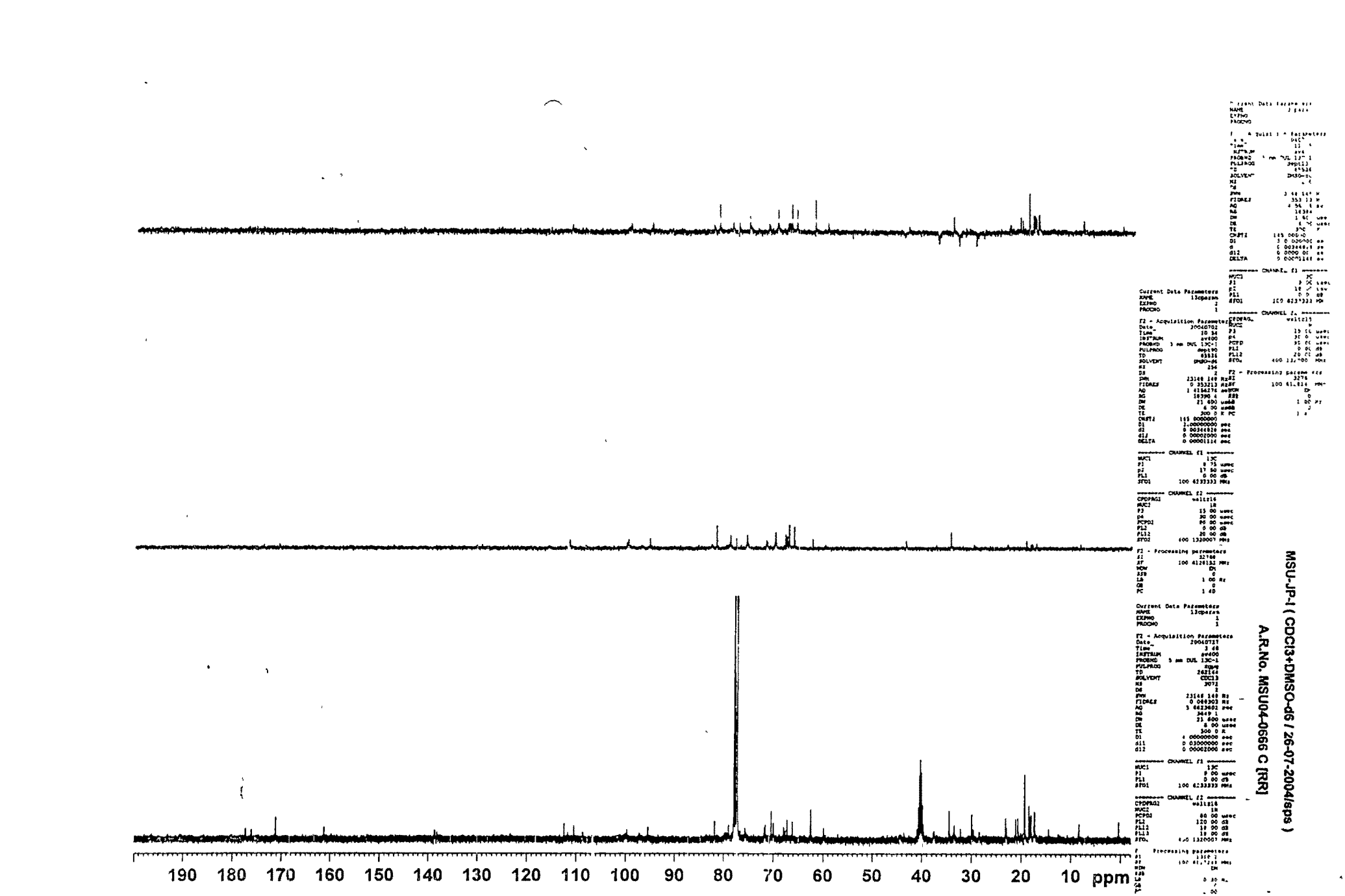
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27	80334.6	7994.989	79.4630	0.18	
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31	80889.8	7896.942	78.4885	0.15	
32	80939.4	7888.175	78.4014	0.18	
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34	81157.0	7849.750	78.0195	13.41	*************
35	81223.3	7838.048	77.9031	1.30	*
36	81339.0	7817.605	77.7000	14.00	**************
37	81521.0	7785.459	77.3805	13.62	**************
38	81677.2	7757.881	77.1064	0.24	
39	81880.0	7722.067	76.7504	0.22	
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46	85404.8	7099.559	70.5632	0.14	
47	85442.1	7092.967	70.4977	0.71	*
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62	102380.9	4101.475	40.7650	0.81	*
63	102500.9	4080.284	40.5544	1.58	**
64	102619.7	4059.295	40.3457	1.96	**
65	102738.3	4038.354	40.1376	1.65	**
66	102857.3	4017.336	39.9287	0.86	*
67	102977.3	3996.149	39.7181	0.39	
68	104109.8	3796.134	37.7302	0.18	

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73	106525.1	3369.580	33.4906	0.35	
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76	108514.0	3018.329	29.9995	0.64	*
77	108585.5	3005.696	29.8739	0.17	
78	108702.6	2985.022	29.6684	0.27	
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83	113559.4	2127.276	21.1432	0.52	*
84	113818.0	2081.610	20.6893	0.54	*
85	114110.8	2029.898	20.1754	0.18	
86	114369.3	1984.240	19.7216	0.17	
87	114572.5	1948.354	19.3649	1.43	*
88	114589.7	1945.320	19.3347	1.62	**
89	115072.8	1860.008	18.4868	0.85	*
90	115173.6	1842.197	18.3098	0.21	
91	115222.6	1833.539	18.2237	0.57	*
92	115336.2	1813.482	18.0244	0.62	*
93	115692.3	1750.588	17.3993	0.70	*
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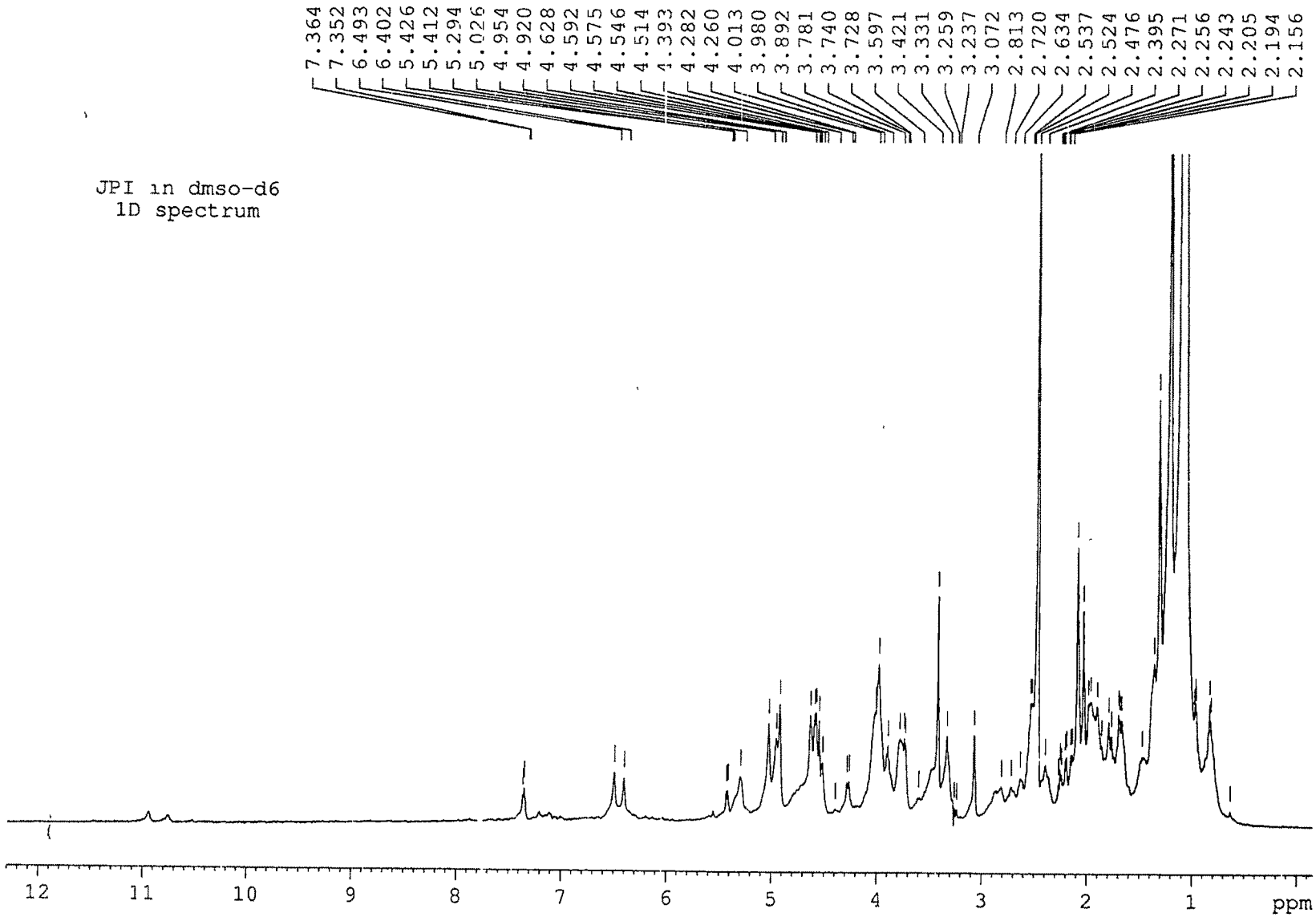

COSY Spectrum of IIIA.

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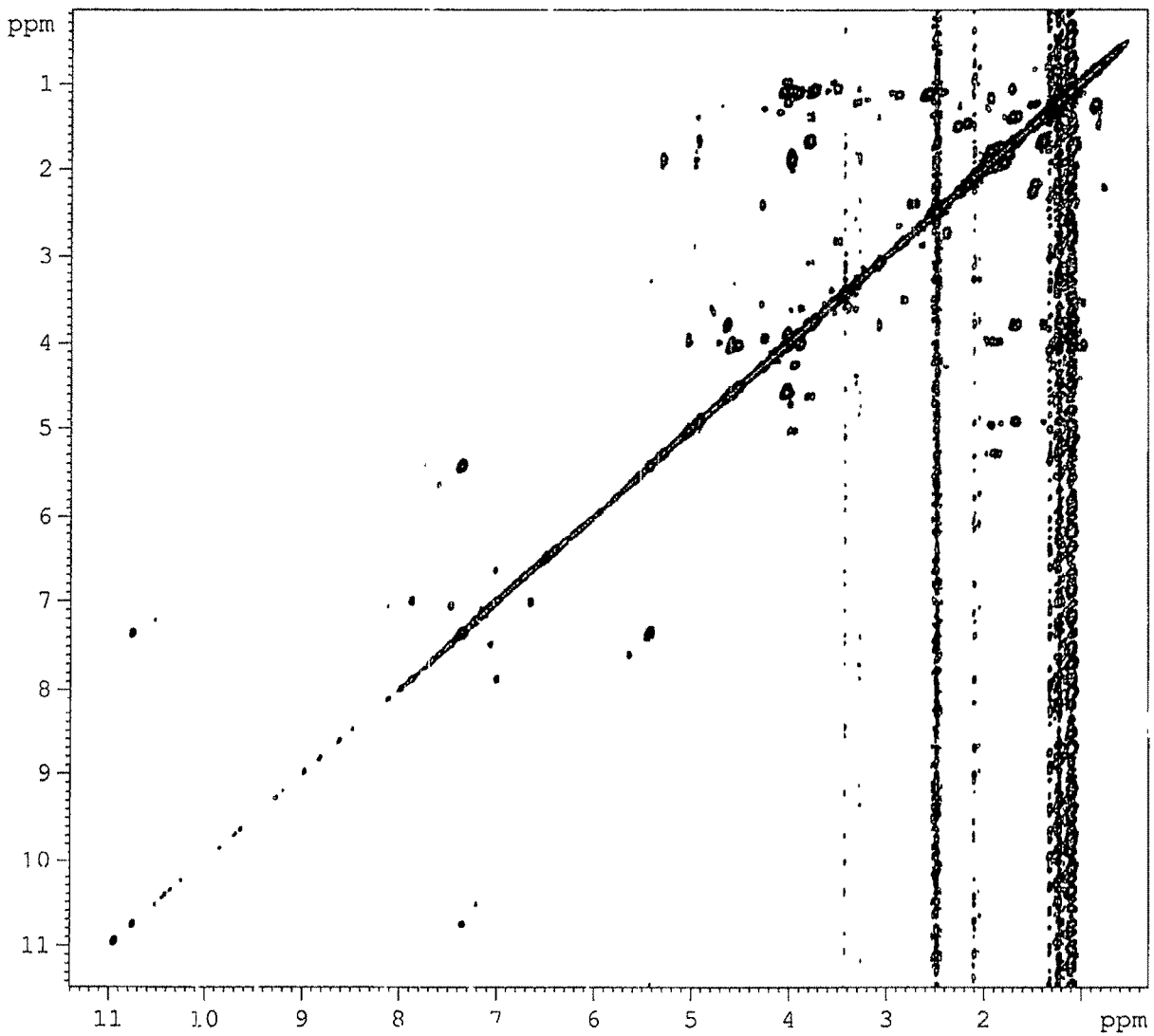
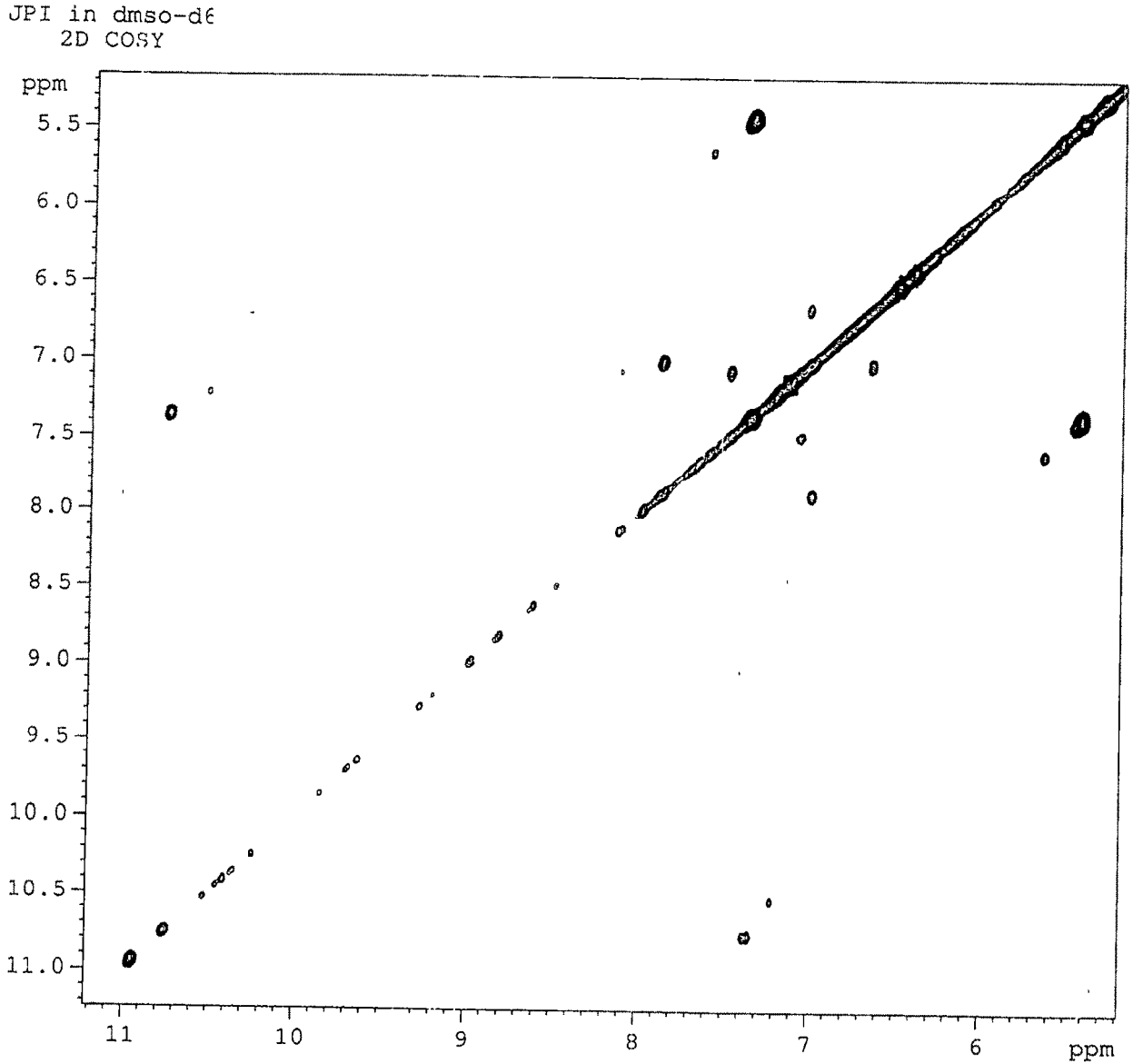
2D COSY

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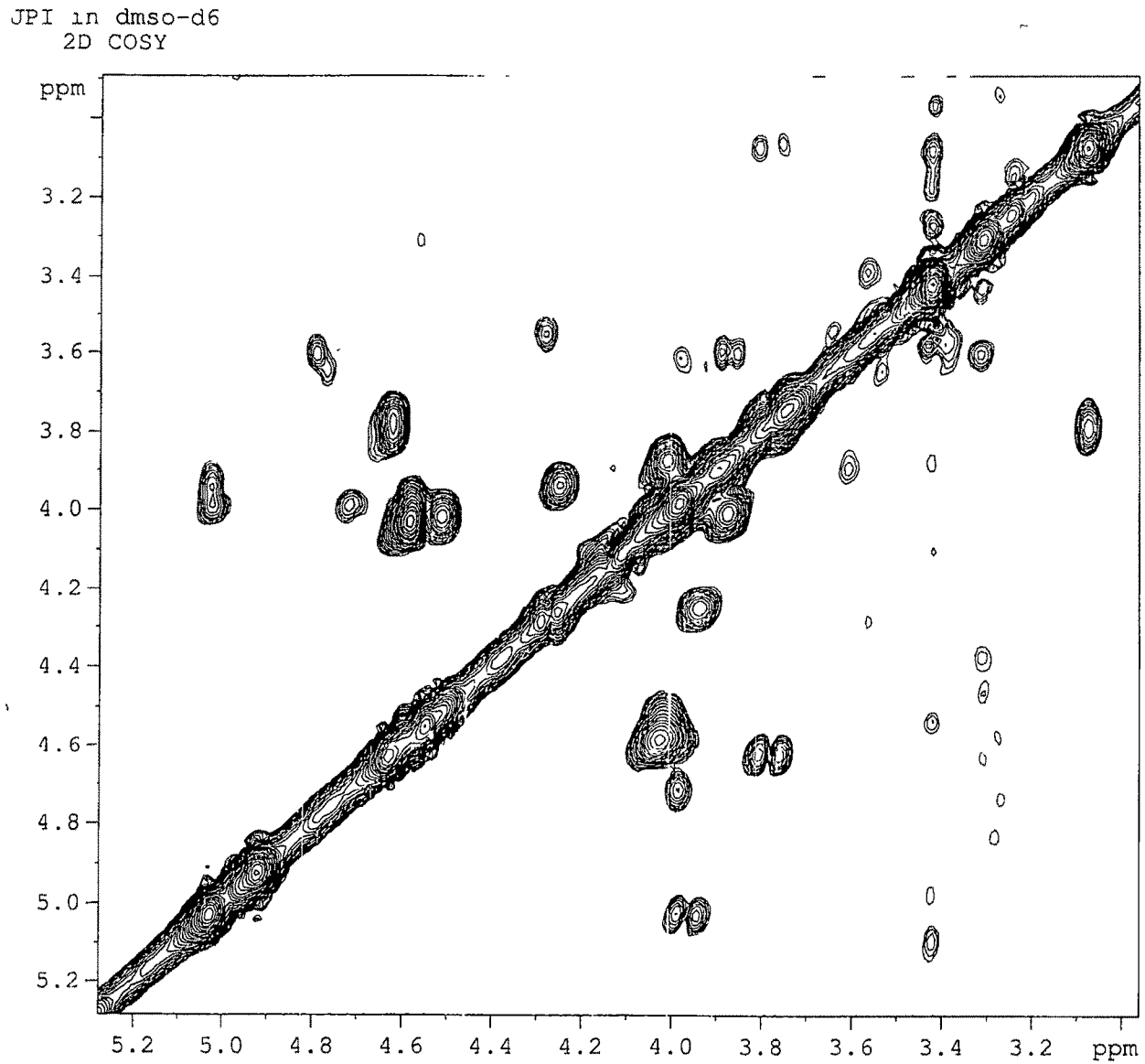
ppm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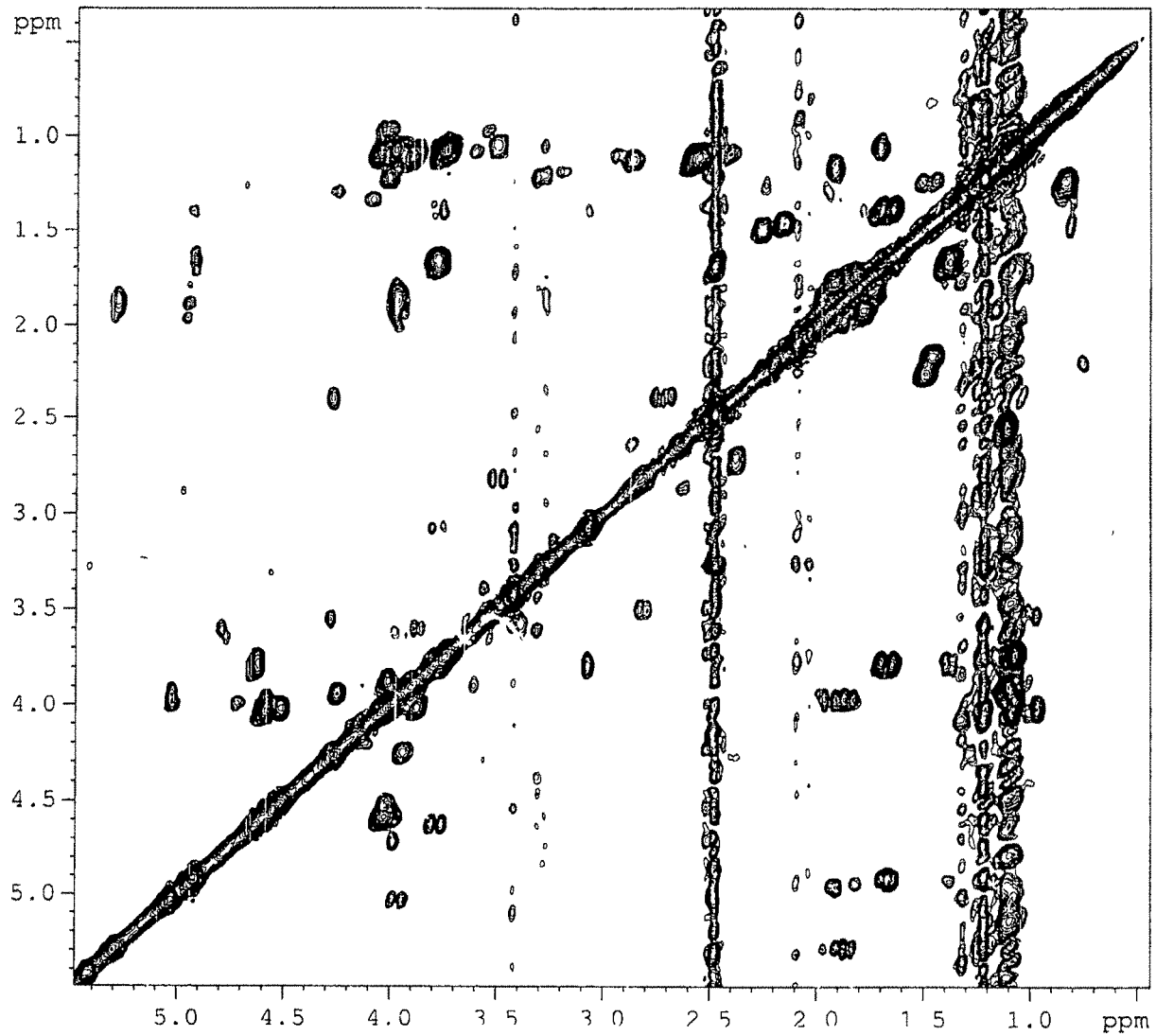
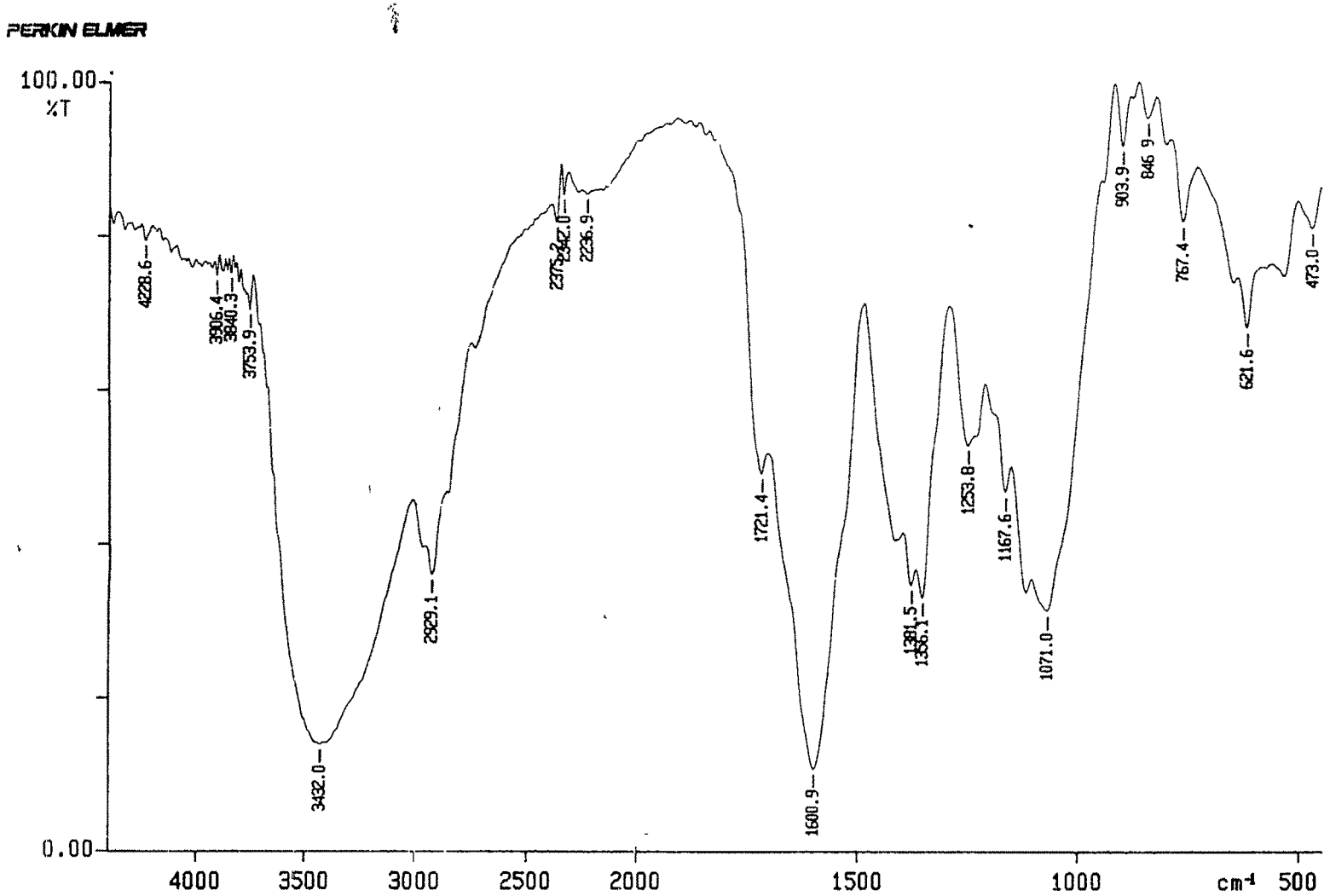
3.2
ppm

JPI in dmso-d6
2D COSY





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SAIF NO - 7852

IR Spectrum of IIIA.

Assessment of the present work in the context of the work done elsewhere: A perspective

Results described in the thesis on characterization of new cluster of PKS genes was undertaken with the aim to further the potential of combinatorial biosynthesis and to understand the rules of PKS programming. The cluster of genes was cloned from a lesser known species of *Streptomyces* precisely for the reason that there is brevity of knowledge in the public domain of the literature of its production potential. In addition, the genetic potential was revealed in terms of production of polyketide compound in possessing the genes for aromatic type II polyketide compound, a finding borne by the genome hybridization to conserved PKS genes. Moreover, *S. flavisclerotícus* also produces multiple bioactivities; thus the happenstance that the polyketide compound is produced is real.

In the straightforward approach to characterization of the PKS genes, PKS genes of the producer organism were screened from the genomic cosmid library. Briefly, the chapters in the thesis describe results obtained with the gene disruption studies (Chapter II), which demonstrate the PKS genes to be expressible and define certain portion of the bioactivity profile. Chapter III includes results obtained with heterologous expression studies, which suggest that the cloned DNA spanning 40 kb does not seem to define complete cluster. Chemical characterization studies reported in Chapter IV identify the polyketide compound as chromomycin.

Chromomycin belongs to aureolic class of compounds, a glycocojugate too, which includes other compounds like mithramycin, olivomycins, chromocyclomycin, UCH9, and durhamycin A, possessing DNA binding properties and described in the literature since 1950. The aureolic acids are neoplastic antibiotics, that act against gram-positive bacteria and also stop the proliferation of tumor cells. In the presence of Mg^{2+}, these compounds inhibit replication and transcription processes by interacting with G-C rich regions in the minor groove of DNA.

The medical applications of the compound are limited due to extreme toxicity in vivo (Gause, 1975). Though the compounds' existence is several decades old, genetic studies of its biosynthesis, regulation are being attempted only recently. Programming of the PKS genes for anguicycline, tetracycline, isochromanequinine, anthracycline class of compounds is being understood, PKS genes of aureolic class of compounds were however not available till recently. Menendez et al, in 1994, cloned the genes for chromomycin cluster from *S griseus* subsp. *griseus* and proposed the biosynthetic pathway for its production from the nucleotide sequence analysis of the cluster. This information will pave way for studies on the PKS genes of aureolic class of compounds (Sanchez et.al., 2005). Furthermore, the cluster is a rich source of glycosyltransferse activities and C- and O-methyl transferase functions. Studies on specificity of these enzyme systems could be exploited for generation of the modified polyketides.

It must be stressed in this context that we could lay claim to be first to sequencing the genes for the chromomycin cluster (Gene data bank Accession No.AY461806, submitted 01-DEC-2003) as against EMBL Nucleotide Sequence Database accession number AJ578458 submitted 15-APR-2005 for the chromomycin sequence from *S. griseus* subsp. *griseus*. Since the identity of the compound was not known then, and the sequencing was partial, the results could not be published.

It is too obvious to ask the question as to how the two clusters compare. Information available presently for ~20 kb of the cluster of *S. flavisclerotícus* indicates that the organization of the PKS cluster of genes is surprisingly conserved between *S. flavisclerotícus* and *S. griseus* subsp. *griseus*, notwithstanding the fact that 16S rDNA ribotyping places the two species quite far apart on the relatedness tree. This conservation warrants further investigation as the closely related mithramycin cluster from *S. argillaceus* is a completely jumbled version of the chromomycin cluster. It would be interesting to study the chromomycin cluster from a new producer species like *S. avellaneus* for conservation of organization of genes. This remarkable conservation means *en bloc* lateral transfer of genes across species resisting rearrangement/swapping.

The gene cluster borne on a mobilizable element could do the trick of horizontal transmission between divergent and distantly related species without the involvement of the rest of the genome. Besides the evolutionary implications, the specific arrangement of genes of the mithramycin and chromomycin clearly did not affect the protein machinery and production of the antibiotic, a fact important for combinatorial biosynthesis (Vinogradova et al., 1975)

Recent studies have shown that similar aromatic polyketide, streptomycin and penicillin gene or portions of clusters are found in otherwise distantly related organisms (Metsa-Ketela, 2002),

Given the hierarchy in the function of the PKS genes, expectedly, KS and CLF genes exhibit the highest similarity index (>90%) with the homologous genes of *S griseus* subsp *griseus*. Genes for modifying functions, on the other hand, are less conserved (50-70%).

However we do present preliminary evidence, which needs to be substantiated, that the genes for resistance may be de-linked from biosynthesis genes in the case of PKS cluster of *S. flavisclerotícus* (see Fig 3.12). This region of the DNA is being sequenced.

Since the availability of the gene cluster for chromomycin production is a recent happening, it is open to genetic manipulation for manifold application.

It has come to the light in the context of the recent results that quite a few species of *Streptomyces* produce chromomycin. Besides *S. flavisclerotícus*, *S. avellaneus* (Kawano et al, 1990), *S. griseus* subsp *griseus* (Menendez, 2004), *Actinomyces aburaviensis* var. *verrucosus* (Vinogradova et al., 1975), *Micromonospora megalomicea* subsp. *nigra* NRRL 3275 (Zazopoulos et al., 2003) etc. reportedly produce chromomycin. Furthermore, the ambiguity in *S. flavisclerotícus* being not classified systematically has been resolved; *S. minutisclerotícus*, known to be producer of chromomycin, has been very recently rechristened as *S flavisclerotícus* (Lanoot et al, 2005).

The state of the art of antibiotic research:

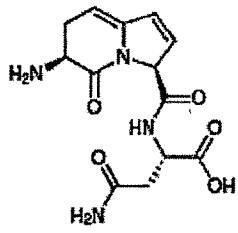
The criteria applied for selecting the organism of the present study did not throw many surprises in terms of the antibiotic being new. In the light of the fact that unexplored and uncharacterized strain of *Streptomyces, S. flavisclerotícus* produces chromomycin a reappraisal the most often asked question is unavoidable – what is the scope for the natural source of microbial world to act as reserve of new antibiotics?

Though in the last two decades, pharmaceutical companies discontinued microbial screening programs mostly for reasons like failure to discover novel molecules; the recent advances however have rekindled efforts in the antibiotic discovery. Some interesting approaches to addressing the question of whether the natural sources should be explored for new leads include a report by Watve et al. (2001) in which the authors estimate the rate of discovery of the new compounds from *Streptomyces* alone, the number of compounds that would make to clinical trials in the search trials spread over five decades, assuming the trend of one new molecule for clinical use for every 20-40 new molecules discovered out of 1000 screened.

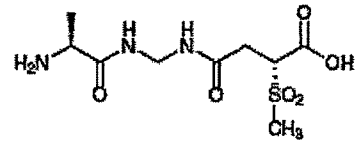
In the same line of thought, an independent estimation by Baltz (2005) enumerated that the frequency of discovery of antibiotics from actinomycetes ranges 10^6-fold in the ~10^7 strains screened and also estimate that less than one part in 10^{12} of the earth's soil surface has been screened for actinomycetes. If the estimates are true, only 1–3% of all *Streptomycete* antibiotics have been discovered. To find the remaining 97–99%, following improvements in the existing strategies are required. A combination of high-throughput screening by modern technologies (10^8–10^9 strains per year), selection against the most common antibiotics, methods to enrich rare and slow-growing actinomycetes, a prodigious microbial collecting and culturing effort, and combinatorial biosynthesis in *Streptomycetes* are some of the approaches. For example, a practical suggestion that *Escherichia coli* K12 derivative designed to harbor 15 antibiotic-resistance genes could select against the most common antibiotics producers of actinomycetes thus enhancing

the signal-to-noise ratio for new molecules with novel modes of action is appealing. (Baltz and Faber, 2006).

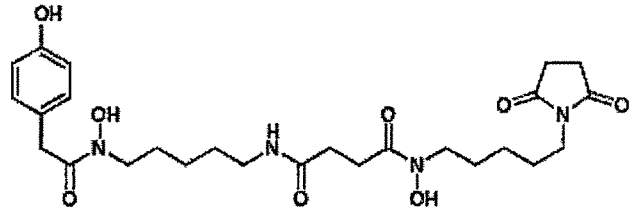
Some of the new antibiotics structures discovered in the recent past by the improved strategies include (i) acyldepsipeptidolactones which suggested that bacterial ATP-utilizing enzymes may be a promising target for antibiotic discovery, (ii) and natural peptidic molecules and lantibiotics targeting lipid II.



Pantocin A



Pantocin B



Terragine A

Efforts to expand the range of bacteria that can be tapped for antibiotic research are being facilitated by several strategies: expanded conventional culturing approaches, novel culture methods, heterologous DNA-based methods and metagenomics. The last in the list of strategies, metagenomics is quite recent. This refers to an attempt to capture DNA from the environment (from the so-called 99% unculturable majority) and use it in heterologous expression systems. Discovery of terragine A and related compounds in a 1,020-member library of soil DNA fragments expressed in *Streptomyces lividans* is described first in the report by Wang et al, (2000). Terragine A is related to the metal-

chelating hydroxamic acids, which are widely produced by bacteria and often show antibiotic activity, however neither terragine A nor any other terragines exhibit antibacterial properties. In 2000, Brady and Clardy reported another approach, featuring much larger libraries (of ~7 × 10^5 metagenomic DNA clones) from soil collected in Ithaca, New York, and an *E coli* expression system.

This approach yielded a series of antibiotic activities due to *N*-acyl tyrosines, a family that differed in the length and degree of unsaturation of the fatty acid acyl groups. This family of antibiotics is the most frequently encountered in *E coli* libraries, and the *N*-acyl tyrosines are produced by a single *N*-acyl synthase.

With the untapped resources aplenty, the need is for ingenuity, innovation and sustained improvements (Clardy et.al., 2006).