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CLASSIFIED BY :

FULL NAME(S) OF APPLICANT(S)

71 INALCO S.p.A

FULL NAME(S) OF INVENTOR(S)

72 MANONI, Marco
SALSINI, Liana
CHINI, Jacopo
CIPOLLETTI, Giovanni

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54 POLYSACCHARIDES DERIVATIVES WITH HIGH ANTITHROMBOTIC ACTIVITY IN PLASMA

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(71) Applicant *(for all designated States except US)*: **ENALCO S.P.A.** [IT/IT]; Via Calabiana 18, I-20139 Milano (IT).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **MANONI, Marco** [IT/IT]; Via Filippo Carcano 10, I-20149 Milano (IT). **SALSINI, Liana** [IT/IT]; Via Gramsci 238, I-55040 Ripa (IT). **CHINI, Jacopo** [IT/IT]; Via Antonio Stoppani, 26, I-50131 Firenze (IT). **CIPOLLETTI, Giovanni** [IT/IT]; Via Tagliamento 21, I-20139 Milano (IT).

(74) Agent: **GERVASI, Gemma**; Notarbartolo & Gervasi S.p.A., Corso di Porta Vittoria 9, I-20122 Milan (IT).

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(54) Title: POLYSACCHARIDES DERIVATIVES WITH HIGH ANTITHROMBOTIC ACTIVITY IN PLASMA

(57) Abstract: The present invention relates to a process for the preparation of sulphated glycosaminoglycans derived from N-acetylheparosan which comprises: a) N- deacetylation and N-sulphation of the N-acetylheparosan polysaccharide prepared from natural or recombinant bacterial strain, preferably K5 E. coli, b) enzymatic epimerization with the glucuronyl C5-epimerase enzyme, c) partial O-sulphation followed by a partial O-desulphation, d) partial 6-0-sulphation, e) N-sulphation and an intermediate step of controlled depolimerization characterised by the fact that both 0-sulphations (0-sulphation and 60-sulphation) are partial. Furthermore the invention relates to the products obtained according to the process which show a ratio between the anti-Xa activity and anti-IIa activity equal to or higher than 1 and to compositions comprising said products in combination with suitable and pharmaceutically acceptable excipients and/or diluent.

WO 2005/014656 A1

Polysaccharides derivatives with high antithrombotic activity in plasma

FIELD OF THE INVENTION

The invention field is the preparation of sulphated polysaccharides with anticoagulant antithrombotic activity starting from polysaccharides with microbial origin.

PRIOR ART

Natural heparin is a polymer having glycosaminoglycanic structure, with variable molecular weights between 3,000 and 30,000 Da, made up of the sequence of repeated disaccharide units made up of uronic acid (L-iduronic or D-glucuronic) and of an amino sugar (glucosamine) bond to each other by β-1-4 bonds. Uronic acid can be sulphated in position 2 and glucosamine can be N-acetylated or N-sulphated and 6-=-sulphated. Furthermore, glucosamine can also contain a sulphate group in position 3.

These substitutes are essential for the creation of the binding region with high affinity to antithrombine (ATIII) and to explicate the anticoagulant and antithrombotic activity of the polymer.

Heparin is the basic anticoagulant and antithrombotic agent for therapeutic use and, even up-to-date, is obtained by extraction from animal organs. In the attempt to substitute this supply source and, therefore, to satisfy the increasing material requests, eliminating at the same time any accidental contamination from infective agents, principally virus or prions, were developed in the last years, vary processes for the preparation of molecules with an heparin-like structure as ell as alike characteristics, starting from N-acetylheparosans polysaccharides having bacterial origin and, therefore, available without quantity limit.

The N.acetylheparosan polysaccharide isolated from a few natural or recombinant K5 Escherichia Coli or from Pasteurella multocida bacterial stocks, has the same basic structure of the natural heparin precursor made up of a repeated sequence of D-glucuronic acid and N-acetyl glucosamine bond to each other by α 1-4 bonds. The bond between the disaccharidic units is, on the contrary, β 1-4.

The uronic acid can be sulphated in two different positions and the glucosamine can be N-acetylated or N-sulphated and 6-O-sulphated. Furthermore glucosamine can contain also a sulphate group in position 3.

The N-acetylheparosan polysaccharide isolated from E.Coli K5 (Vann W.F., Schmidt M.A., Jann B., Jann K. (1981) in Eur. J. Biochem 116, 359-364) was chemically modified as described by Lomeau et al. in US patent n. 5,550,116 and by Casu et al (Carb. Res 263-1994-271-284) or chemically and enzymatically in the attempt to obtain products endowed with biological activity comparable to the one of extractive heparin.

Furthermore the semi synthetic products must undergo a depolymerization process to decrease molecular weight which makes the product more suitable in the different therapeutics applications in particular it improves the bio availability and reduce the bleeder risk associated to their use and other side effects.

Chemicals and enzyme modifications of the bacterial polysaccharide are described for example in the Italian patent N° IT1230785 where the K5 polysaccharide is N-desacetylated and N-sulphated; then it undergoes C5 enzymatic epimerization of the glucuronic acid. These transfers are followed by other transfers of enzymatic sulphation both on uronic acid and on the aminosugar.

The patent application WO92/17509 describes a method for heparin like products preparation starting from K5 polysaccharide by means of N-desacetylation, N-sulphation and C5 enzymatic epimerization passages, followed by chemical O-sulphation and optionally by a N-sulphation.

The patent application WO 96/14425 and the patent US n° 5,958,899 describe a method for the preparation of K5 polysaccharide derivatives having high content of iduronic acid obtained by N-desacetylation and N-sulphation, enzymatic epimerization to iduronic acid of more than 50% of the glucuronic acid using modified buffers to obtain a critical viscosity, followed by sulphation of at least some of the free hydroxyl groups of the uronic acid and of the glucosamine groups.

The patent application WO97/433117 and the patent US 6,162,797 describe the preparation of K5 derivatives with high anticoagulant and antithrombotic activities obtained by means of N-deacetylation and N-sulphation, enzymatic epimerization of the glucoronic acid, and by O-supersulphation and N-risulphation.

The patent application WO 98/42754 and the patent US n° 6,197,943 and Naggi A. et al. Carbohydrate Research 336 (2001) 283-290, describe a methodology for the preparation of sulphated glycosaminoglycans including K5 polysaccharide derivatives having high antithrombotic in vitro activity, by means of solvolitic desulphation of supersulphated precursors and optional 6-O risulphation.

The patent application WO 0172848 and WO 02/50125 describe a method of preparation of glycosaminoglycans derivative from K5 polysaccharide having high anticoagulant and antithrombotic activity. The process comprises the following passages: a) N-deacetylation b) N-sulphation c) enzymatic epimerization of the glucuronic acid in iduronic acid d) supersulphation e) partial chemical desulphation f) optional selective 6-O risulphation. The process is characterized by the use of a C5 glucuronil epimerase enzyme in a truncated form, in solution or immobilized.

Moreover, the patent application US n° 09/732,026 and Li et al. J. Biol. Chem, vol 276, 213 (2001) 20069-20077 have led to the discovery of a new mouse gene for the expression of the C5 epimerase enzyme containing the additional sequence at the N-terminal end which allow the production of complete forms of the enzyme having higher activity/stability with respect to the former.

SUMMARY

The present invention refer to a process for the preparation of sulphated glycosaminoglycans derived from acetyl N-heparosane which comprises the following steps:

a) N-deacetylation and N-sulphation of a N-acetylheparosan polysaccharide isolated from natural or recombinant bacterial source,

b) enzymatic epimerization through the C5-epimerase glucuronil enzyme,

c) partial O-sulphation combined to a partial O-desulphation,

d) partial 6-O sulphation

e) N-risulphation

further comprising an intermediate controlled depolymerization step carried out alternatively after step b), c) or d) and wherein such process is characterized by the fact that the partial O-sulphation in step c) is carried out using a molar ration between the sulphating/hydroxyl agent of the N-acetylheparosan lower than or

equal to 5, more preferably lower than 2.5 or even more preferably lower than 1.5 and that the partial 6O-sulphation in step d) is carried out using a molecular ratio between the sulphating/hydroxyl agent of N-acetyl heparosan equal to or lower than 2.

According to a preferred embodiment the intermediate depolymerization is carried out after the epimerization step b).

Both partial O-sulphation and partial 6O-sulphation are carried out with sulphating agents selected among: triethylamine-SO_3, trimethylamine-SO_3, pyridine-SO_3 in an aprotic polar solvent preferably non-donors of formyl groups, such as tetramethylen-sulfone, 2,4-Dimethylsulfolane, N,N,-Dimethylacetamide or N,N,-diethylacetamide. Optionally the process comprises an affinity selection step on a matrix carrying antithrombine III or its fragments.

The invention also concerns the sulphated glycosaminoglycans K5OS6OSNS-epi obtained according to the described process for pharmaceutical use. These products are characterized by a 6O-sulphation degree higher than 40% and preferably comprised from 50% to 85% very close to the values of extractive heparin and by the presence, at the reducing end, of a anhydrous mannitol residue, preferably sulphated in positions 1, 3 and 6. They are also characterized by a sulphation degree of the hydroxyl group in position 1 and 6 of the anhydrous mannitol equal to or higher than 20% and according to a preferred aspect by the complete absence of formyl groups on the amino sugar.

According to the invention the sulphated glycosaminoglycans show a biological activity anti-factor Xa in plasma higher than that of the biotechnological heparins obtained according to prior art methods and a ratio between antiXa and anti IIa activity equal to or higher than 1, alike the extractive heparins.

The products obtainable according to the process of the invention:

a) are able to release a tissue factor inhibitor (TFPI) from the cells of the vascular endothelium just like the extractive heparins or even more,

b) are particularly resistant to degradation with hydrolythic enzyme such heparinase I,

c) are able to inhibit thrombin and Xa factor protease release,

d) show a low affinity for the PF4 factor (platelet factor 4).

According to a further aspect, the invention refers to biotechnological heparins (modified N-acetyl heparosans) obtained according to the process of the invention for therapeutic use and to the pharmaceuticals compositions comprising such products as active principles. According to a further aspect the invention regards the use of the products obtained for the preparation of drugs with antithrombotic and anticoagulant heparin-like activities, for the preparation of profibrinolytic and anti-aggregant medicaments and for the preparation of medicaments for the prophylaxis and the treatment of thrombo-embolytic disorders caused by congenital or acquired lack of antithrombine III.

A further aspect of the invention concerns the preparation of O-sulphated, K5OSNH$_2$-epi and K5OS6OSNH$_2$-epi intermediates carrying the amminic group of the amino sugar free, preferably free from formyl groups wherein such intermediates can be isolated and used for the preparation of N-sulphated and/ or N-acetylated heparosan derivatives.

DESCRIPTION OF THE DRAWINGS

Fig. 1 spectrum ^{1}C-NMR of the anomerical region of the polysaccharide K5 N-sulphate- epimerised as described in example 1.

Fig. 2 spectrum ^{1}H-NMR of the product obtained in example 1.

Fig. 3 spectrum ^{1}H-NMR of the product obtained in example 2.

Fig. 4 spectrum ^{13}C-NMR of the product obtained in example 3.

Fig. 5 spectrum ^{13}C-NMR of the product obtained in example 4.

Fig. 6 spectrum ^{13}C-NMR of the product obtained in example 5.

Fig. 7 spectrum ^{13}C-NMR of the product obtained in example 6.

Fig. 8 spectrum ^{13}C-NMR of the product obtained in example 7.

Fig. 9 spectrum ^{13}C-NMR of the product obtained in example 8.

Fig. 10 spectrum ^{13}C-NMR of the product obtained in example 9.

Fig. 11 spectrum ^{13}C-NMR of the product obtained in example 10.

Fig. 12 spectrum ^{13}C-NMR of the product obtained in example 11.

Fig. 13 spectrum ^{13}C-NMR of the product obtained in example 12.

Fig. 14 spectrum ^{13}C-NMR of the product obtained in example 13.

Fig. 15 spectrum ^{13}C-NMR of the product obtained in example 14.

Fig. 16 spectrum ^{13}C-NMR of the product obtained in example 15.

Fig. 17 spectrum ^{13}C-NMR of the product obtained in example 16.

Fig. 18 spectrum ^{1}H-NMR of the product obtained in example 18.

DETAILED DESCRIPTION OF THE INVENTION

According to a main aspect, the invention is related to a process for the preparation of sulphated glycosaminoglycans derived from N-acetylheparosan and called for the purpose of this invention, "Biotechnological Heparins", which comprises the following steps:

a) N-deacetylation and N-sulphation of a N-acetylheparosan polysaccharide isolated from a natural or recombinant bacterial source,

b) enzymatic epimerization by a glucuronyl C5-epimerase enzyme,

c) partial O-sulphation combined to partial O-desulphation,

d) partial 6-O selective sulphation,

e) N-risulphation,

wherein this process furhter comprises an intermediate step of controlled depolymerization carried out alternatively after step b) or c) or d) and wherein said process is characterized by the fact the O-sulphations are partial, wherein in step c) this is achieved using a molar ratio between the sulphating agent and the substrate hydroxyl groups (epimerised N-acetyl heparosan) lower than or equal to 5, more preferably lower than or equal to 2,5 or, even more preferably, lower than or equal to 1.5 and a sulphation time lower than 10 hours. A partial 6O-sulphation according to step d) is obtained by using a molar ratio between the sulphating agent and the hydroxyl groups of the substrate (epimerised heparosan N-acetyl) lower than or equal to 2, or more preferably lower than or equal to 1,5 and sulphation time lower than 2 hours or, even more preferably lower than or equal to 90 minutes, or, even more preferably, lower than or equal to 60 minutes at a temperature comprised between 4°C and 30°C, preferably between 10°C and 25°C.

The partial O-sulphation according to step c) and the partial 6O-sulphation according to step d) are carried out with known sulphating agents in an aprotic polar solvent preferably non-donor of formyl groups, more preferably selected from: N,N, dialkylacetamide (more preferably N,N,-Dimethylacetamide or N,N,

diethylacetamide) and sulpholans (preferably tetramethylen-sulfone or 2,4-Dimethylsulfolane).

The use of an organic solvent non donor of formyl groups combined to partial sulphation conditions leads to products characterized by the lack of formyl groups or their derivatives on the amino sugar and to a distribution of sulphate groups similar to the one of extractive heparins.

According to the process of the invention, controlled depolymerization is carried out as an intermediate step, which means alternatively after step b), c) or d) and not in a final phase as described in the prior art. It is preferably carried out on the epimerised N-sulphate heparosan polysaccharide before or after step c) of partial O-sulphation. It can be carried out by physical methods including a gamma rays treatment or by chemical methods including a beta-gamma treatment with nitrous acid or its salts or a treatment with periodic salts or a free-radicals treatment. According to a preferred aspect, the depolymerising agent is nitrous acid and the polysaccharide is used in a quantity comprised from 1 to 100 mg salt/g of polysaccharide. The reaction is performed at a temperature comprised from 4 to 10°C. More preferably, controlled polymerisation is carried out for less than 30 minutes in the presence of sodium nitrate and it is terminated by adding a molar excess of borohydride sodium.

The intermediate depolymerization allows to obtain a low molecular weight product, preferably with a molecular weight lower than or equal to 15000 Da, more preferably comprised from 3000 to 90000 Da, carrying an anhydromannitol residue at the reducing end which shows, besides the hydroxyl sulphation in position 6, like in the extractive heparins, the hydroxyls sulphation in position 1 and 3.

However the process is compatible also with a further depolymerization carried out at the end of the process. It has been observed that, when the depolymerization is carried out in an intermediate phase, the final products have anticoagulant and antithrombotic activity and a ratio between antiXa and anti IIa, unexpectedly higher than those found in products with the same molecular weight but obtained after a depolymerization performed after sulphation/desulphation and 6O-sulphation steps, as demonstrated by the data shown in table 1.

According to a preferred process embodiment, the N-acetyl polysaccharide heparosan is preferably derived from E.coli K5.

The process can further and optionally comprise a final phase of enrichment of the products resulting from steps a)-e), consisting in an affinity-chromatography on antithrombine III as described in Hook et al. FEBS Lett 1976, 66:90-93.

N-deacetylation and N-sulphation are carried out according to prior art methods which comprise an alkaline hydrolysis performed at a temperature comprised from 30 to 80°C, preferably from 40 to 60°C, for a period of time comprised between 10 and 30 hours, preferably between 15 and 20 hours, followed by a treatment, for a time of up to 12 hours at 20-65°C with a sulphating agent, preferably pyridine-sulphotrioxide in sodium carbonate.

The epimerization in step b) is carried out with the natural or recombining glucuronil C5 epimerase enzyme preferably in an immobilized form.

The enzyme is preferably the recombinant one described in WO98/48006 or even more preferably the one described in US n°09/732,026 and is preferably expressed and purified from insect cells or from yeast strains such as for example Saccharomyces Cerevisiae, Pichia methanolica, Hansenula polymorpha, Saccharomyces pombe, Kluyveromices lactis, Kluyveromices lactis, Kluyveromices fragilis.

The enzyme immobilization is preferably carried out on resins CNBr Sepharose 4B (Pharmacia) or polymethacrylic or polystyrenic resins, with epoxidic groups or diolic groups activated with CNBr, in buffer NaHCO3 100-300 mM or in phoshate buffer 10-50 mM at pH 7.0-8.3, more preferably at pH 7.2-7.8, at a temperature of 4-25° for 12-72 hours.

According to a more preferred aspect, the epimerization reaction occurs at a temperature not higher than 35°C, preferably at a temperature comprised from 15 to 30°C, more preferably comprised from 20 to 25°C.

The epimerization is performed according to known methods, such as the ones described in WO 01/72848, preferably at a temperature not higher than 35°C, more preferably between 15 and 30°C, or even more preferably between 20°C and 25°C.

The epimerization buffer is preferably a HEPES solution (preferably in concentration 25 mM) with pH comprised between 5,5-8.0, more preferably between pH 6,5-7,0, and further comprises the N-deacetylated and N-sulphated polysaccharide, EDTA 10-30 mM, preferably 15-25 mM, $CaCl_2$ (or alternatively salts of other divalent cations such as Zn^{2+} , Ba^{2+}, Mg^{2+}, Mn^{2+},) in concentration comprised between 70 and 150mM, much more preferably between 75-100 mM. The solution is thermostated at a temperature comprised between 15° and 30° C (preferably 20-25° C), preferably recycled at a flow of 30-240 ml/hour, for a time between 1 and 24 hours. The column contains preferably from $1,2 \times 10^7$ to 3×10^{11} cpm equivalents of the immobilized enzyme on inert thermostated support.

The above defined operative conditions, in particular the preselected temperature, stabilize the epimerase C5 enzyme for thousand hours, allowing a remarkable saving of time and of the reagents for the preparation of the epimerization column.

The partial O-sulphation in the process (step c) is performed by using known sulphating agents such as triethylamine-SO_3, trimethylamine-SO_3, pyridine-SO_3 in an aprotic polar solvent, preferably non-donor of formyl groups. It is performed using a molar ratio between the sulphating agent and the substrates hydroxyl groups lower than or equal to 5 or preferably lower than or equal to 2,5 or more preferably lower than 1,5, for a period of time equal to or lower than 10 hours, more preferably equal to or lower than 8 hours, preferably comprised from 1 to 6 hours at a temperature from 20 to 70°C, preferably from 30 to60°C.

The partial O-sulphation is followed by a partial desulphation performed by treatment with a desulphating agent such as DMSO in methanol, for a period of time comprised from 10 to 240 minutes at a temperature comprised from 45 to 90°C.

Each process step can also comprise precipitations and/or intermediate polysaccharide desalifications according to known methods.

The partial 6O-sulphation (step d) is obtained by adding a sulphating agent in molar ratio with the substrate hydroxyl groups equal to or lower than 2 or preferably lower than 1,5 for a period of time equal to or lower than 2 hours, or preferably equal to or lower than 90 minutes, even more preferably between 4°C and 30°C, preferably 10°C and 25°C in solution with an aprotic polar solvent,

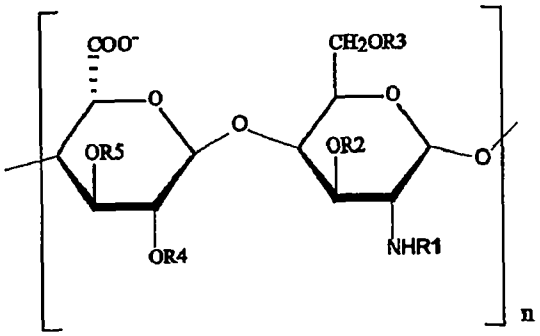
preferably non donor of formyl groups. According to an alternative embodiment of the process, the partial 6O-sulphation (step d) is performed after the N-risulphation and steps d) and e) are performed in an inverted order.

The N-risulphation (step e) is preferably performed in carbonate buffer by adding a known sulphating agent, such as for example trietylamine-SO_3, trimethylamine-SO_3, pyridine-SO_3.

In conclusion, the process of the invention shows the following innovative elements: partial O-sulphation (O-sulphation and 6O-sulphation), depolymerization performed in an intermediate and not as a final step and, furthermore, a partial O-sulphation and 6O sulphation performed in a aprotic polar organic solvent preferably non-donors of formyl groups.

The N-acetyl-heparosane derivatives obtained from the process of the invention present characteristic structure and biological differences with respect to the biotechnological heparins obtained according to well-known prior art processes.

From a chemical point of view the polysaccharides of the invention are defined as a polysaccharidic chain mix represented by the following general formula (I)



Formula (I)

where n ranges from 3 to 150, R1 can be a hydrogen, a group SO3 or an acetyl group. R1 does not present other functional groups such as formyl groups. R2, R3, R4 and R5 can be hydrogen or a SO3- group where preferably R1, R2, R3, R4, R5 are substituted as follows:

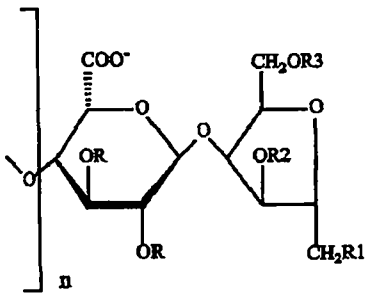
- R1 from 85 to 97% with $SO3^-$ groups and/or from 3% to 15% of acetyl groups and/or from 0 to 12% of H^+

- R2 from 15 to 60% with $SO3^-$
- R3 with $SO3^-$ groups to at least a 40%, preferably from 50% to 85%
- at least 20% of the glucuronic acid units are non sulphated in positions R4 and R5.

In particular the depolymerised polysaccharides according to the process of the invention, show at their reducing extremity an anhydromannitol residue with one ore more of sulphated hydroxyl.

This occurs when the depolymerization is carried out in presence of nitrous acid or its derivatives, such as the sodium nitrite, followed by the borohydride sodium treatment, to obtain the compounds according to the following structure (II)

Formula (II)



where R1, R2, R3 can be hydrogen or a $SO3^-$ group and preferably where :

- R1 ranges from 0 to 100% of $SO3^-$
- R2 from 0 to 100% of $SO3^-$
- R3 from 0 to 100% of $SO3^-$

Preferably R1 and R3 comprise from 20% to 85% of $SO3^-$ and R2 from 15 to 60% of $SO3^-$

The preferred products are those with a molecular weight lower than or equal to 15000 Da, or preferably comprised from 1500 and 15000 Da, even more preferably from 3000 to 9000Da.

The products obtained according to the invention are different from a structural point of view as compared to the products of the prior art, because of the presence of multiple signals in the ^{13}C NMR spectrum region comprised from ppm 79 to 89,

in particular from 80 to 86 ppm, which are in excess with respect to the characteristic signals of the anhydromannitol (see figure 10 which shows the ^{13}C NMR spectrum at high resolution of the sample prepared in example 9) and which indicate the presence of anhydromannitol variously sulphated in particular on the hydroxyls in position 1 and 6, in low molecular weight products prepared according to the process of the invention with an intermediate depolymerization.

More particularly the products obtained according the invention process are different because of sulphation of the hydroxyls in position 1 of the anhydromannitol as showed by the increase of the signal in the region at ppm 67-68 and by the decrease of the signal disappearance in a region at ppm 61-63 in spectrum ^{13}C NMR.

The difference is showed by comparing the spectrum of figure 11 (corresponding to the polysaccharide produced as described in example 10) and the one shown in figure 9 (polysaccharide produced as described in example 8). These differences are more evident by means of two-dimensional NMR according to the method described in Guerrini et al. Seminars in Thrombosis and Hemostasis, vol 27, 5, 473-482, 2001.

The main characteristic of these regions derive from the partial or total sulphation of the available hydroxyl groups of the anhydromannitol which are formed at the reducing extremity of the polysaccharide during depolymerization, when this is carried out before the sulphation step and in particular by the sulphation of the hydroxils in position 1 and 6.

A further characteristic of the products of the invention with respect to the products and procedure of the prior art (such as i.e. the ones showed in the experimental example 6 and 7 comparative of the present application) is the absence of the signals at 7-9.5 ppm in the spectrum ^{1}H NMR and at 51 and 165 ppm in the spectrum ^{13}C NMR which indicates the absence of chemicals groups different from a free aminic group or from an acetyl group or from the sulphate group on the glucosamine in all the products derived with low and high molecular weight.

On the contrary these signals are usually present in products derived from processes wherein sulphation of N-acetyl heparosan is performed in organic solvents donors of formyl groups such as N,N, dialkyl formamides.

Sulphated glycosaminoglycans obtained according to this invention show an anticoagulant activity measured as anti-factor Xa in presence of plasma, higher than that of biotechnological heparins obtained according to known modification methods. Moreover, they show a ratio between antiXa and anti IIa activity equal to or higher than 1, very similar to the one of extractive heparins.

In particular the new products show:

a) a factor Xa inhibitory activity higher than 50IU/mg, more preferably higher than 70 IU/mg in tests carried out in the presence of human plasma. The anti Xa factor activity is preferably measured as described in Ten Cate H et al. Clin. Chem 3,860-864 (1984) or in European Pharmacopoeia 1997 3rd edition. In the presence of plasma this biological activity is surprisingly higher than that of biotechnological heparins produced with prior art processes, and is similar to the one measured for heparins extractive with high or low molecular weight,

b) an activation capacity of the TFPI (Tissue Factor Pathway Inhibitor, described in Bronze GJ Jr et al. Blood 71, 335-343, 1988) equal or higher than that of extractive heparins,

c) a ratio between anti Xa/anti IIa activities equal or higher than 1 at comparable molecular weight. More preferably the ratio is higher than 1.5,

d) a resistance to heparinase I digestion equal or higher than that of extractive heparins,

e) capacity to inhibit the thrombin and Xa factor protease release,

f) low affinity for the PF4 factor (platelet factor 4)

The activation ability of TFPI from vascular endothelial cells enhance the antithrombotic and antinflammatory activity of these products and extends and improves the therapeutic indications to deep venous thrombosis in chirurgical procedures, ischemic complications of the unstable angina and to myocardial heart attack and to ischemic events.

The ability to inhibit the protease production, combined to the increased TFPI production, allows the further extension of the therapeutic indications of these products to the treatment of sepsis and of its complications such as the

disseminated intravascular coagulation (CID) and to the treatment of diseases caused by congenital or acquired lack of antithrombine III.

The determination of the activity on TFPI factor after treatment with the products of the invention is carried out for instance *in Vitro* on HUVEC cells according to the process described in Gory AM. Et al. Thromb. Haemostasis:81:589-593 (1999).

The N-acetyl heparosan derivatives obtained according to the invention (biotechnological heparins) are particularly resistant to degradation with hydrolytic enzymes such as the heparinase I. This characteristic together with the possibility to obtain low molecular weight products that by itself increases the bioavailability and decreases the hemorrhagic risk associated to their use and the collateral effects with respect to high molecular weight heparins, together with a high Xa anti-factor activity and with a low sulphation degree, allows their use not only for the parenteral but also for the oral way of administration.

As before mentioned, the product obtained with the process invention show the capacity to inhibit the thrombin protease generation and Xa factor.

The inhibition of proteases generation is preferably carried out in fibrinogen-depleted plasma. The inhibition of both the thrombin (factor II) and the Xa factor generation is preferably monitored using an amidolytic method both for the intrinsic and extrinsic coagulation system.

According to both methods, in both the systems used, the products derived from the invention show a strong inhibition activity both of the thrombin and of the Xa factor and this characteristic improves the antithrombotic profile of these products.

The products obtained according to the process of the invention are furthermore endowed with a low affinity for the PF4 factor (Platclet Factor 4) that can be measured in plasma as residual anti-Xa activity after adding addition of a fixed quantity of PF4 factor in the solution containing the biotechnological heparins.

The residual anti Xa activity calculated as percentage with respect the initial activity is higher than the one obtained with extractive heparins or with extractive heparins with low molecular weight which indicates a lower affinity for PF4.

This lower binding affinity improves the clinical profile of the products obtained according to the invention as it decreases the risk of thrombocytopenia onset induced from heparin (HIT). Even if the preferred molecular weight of the

obtainable products is lower than 15000 Da, or is more preferably comprised from 3000 to9000 Da, molecular weight products >15000 Da are obtainable simply by changing the depolymerization conditions, still maintaining their biological properties such as the anti-Xa high activity, the heparinase resistance and the factor TFPI liberation .

In conclusion the biotechnological heparins produced according to the invention present the following main characteristics:

- a region comprised from 79 to 89 ppm or more precisely comprised from 80-86 ppm by ^{13}C NMR characterized by the presence of multiple signals in excess with respect to the characteristic signals of the anhydromannitol and a signal increase at 67-68 ppm and/or a the signal decrease or even disappearance at 61-62 ppm by ^{13}C NMR. These signals indicate the presence of variously sulphated anhydromannitol, In particular sulphated in position 1 , 3 and 6 and, even more particularly sulphated on the hydroxyl in position 1, as emphasized in the comparison between the spectra of figure 11 and of figure 9.
- preferably the absence of signals at 7-9.5 ppm in a ^{1}H NMR spectrum and the absence of signal at 51 and 165 ppm in ^{13}C NMR spectrum which indicates the absence of formyl groups.
- a anti-Xa (anticoagulant) activity in plasma higher than the one of biotechnological heparins prepared according to prior art methods.
- a anti-Xa activity/anti IIa factor ratio equal or higher than the one of biotechnological heparins prepared according to the prior art methods, preferably higher or equal to 1 or much more preferably higher or equal to 1.5.
- a resistance to heparinase equal to or higher than extractive heparins;
- the ability to inhibit thrombin and Xa factor production
- low PF4 affinity

The biological activities of the newly produced biotechnological heparins are peculiar: in particular a ratio between the anti-Xa activity and the anti-IIa activity equal to or higher than 1, it is usually in products obtained according to prior art is lower than 1 indicating an optimal ratio between the antithrombotic and anticoagulant activities which results from APTT values. Such ratio is similar to the one of extractive heparins.

An optimal characteristic also in respect to extractive heparins, evaluable from the high HCII values, is a better direct inhibition of thrombin, which implies the possibility to use the products of the invention in thrombo-embolytic and/or vascular disorders due to thrombin and in acquired or congenital lack of antithrombin III.

According to a further aspect the invention concerns the use of the products obtained according to the process described, alone or formulated in compositions with suitable pharmaceutically acceptable excipients or diluents, for the anticoagulant and antithrombotic treatment or the prophilaxis in substitution of extractive heparins and for the preparation of pharmaceuticals with profibrinolytic and antiaggregating activity.

Particularly suitable is the use of the invention products or of compositions comprising such products as the active ingredient for the prophylaxis and the treatment of unstable angina, myocardial heart attack, deep venous thrombosis, lungs embolism, ischemic events as well as for the treatment of sepsis and for the prevention of its complications such as disseminated intravascular coagulation (CID).

According to a further aspect the invention concerns the use of the invention products for the preparation of pharmaceuticals for the prophylaxis and treatment of unstable angina, arterial thrombosis, atherosclerosis and for the treatment of thromboembolic diseases due to congenital or acquired lack of antithrombin III.

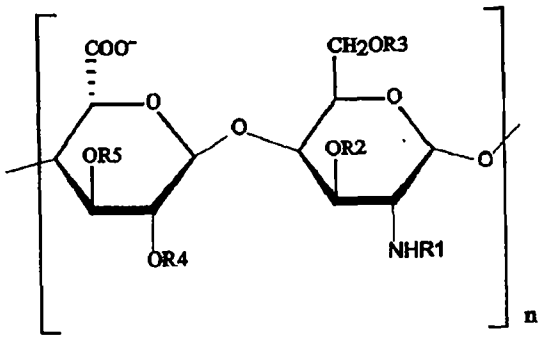
The products can be carried by micelles, carrier molecules etc. and result particularly suitable for oral use besides for the parenteral one.

Therefore it represents a further aspect of the invention the pharmaceutical compositions containing as the active principle the polysaccharides derivatives of the N-acetyl-heparosans produced according to the process of the invention, in appropriate formulations both for the oral and the parenteral use.

According to a further aspect the invention concerns the preparation of O-sulphated intermediates with the free amminic group of the amino sugar and completely lacking formyl groups and without any of anticoagulant activity, useful, for example, in the preparation of the final products of the invention.

Particularly preferred is the intermediate K5-OS,NH_2,epi obtained and isolated according to the process described in this invention, which is defined as a mixture of polysaccharidic chains represented by the following general formula (III):

Formula (III)



where n ranges from 3 to 150, R1 can be hydrogen or an acetyl group with an the acetylation degree ranging from 3% to 15%.

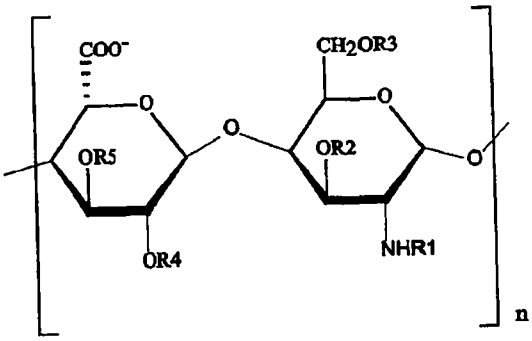
R1 does not carry any other functional groups, preferably it does not carry formyl groups; R2, R3, R4 and R5 can be hydrogen or a SO3- group wherein sulphation range is preferably comprised from 30% to 98%.

K5OSNH$_2$-epi, (^{1}H NMR spectrum shown in figure 16) has a molecular weight preferably comprised between 1500 and 15000 Da or, more preferably from 3000 to 9000 Da and is characterised by an anticoagulant activity lower than 10 IU/mg as measured by the anti factor Xa activity with a chromogenic method (Coatest Heparin kit, Chromogenix).

K5OSNH$_2$-epi is useful, for example, in the preparation of the products of the invention.

K5-OS6OSNH$_2$,epi is obtained and it can be isolated according to this invention is defined as a mixture of polysaccharidic chains represented by the following general formula (IV):

Formula (IV)



where n ranges from 3 to 150, R1 can be hydrogen or an acetyl group where the acetylation range is comprised from 3% to 15%. R1 preferably does not carry other functional groups. Preferably it does not carry formyl groups.

R2, R3, R4 and R5 can be hydrogen or a SO3- group where R2, R3, R4, R5 are preferably substituted as follows:

- R2 from 15 to 60% of SO3-
- R3 higher than 40%, preferably from 50 to 85% of SO3-

and at least 20% of glucuronic acid units are not sulphated in positions R4 and R5.

K5-OS6OSNH$_2$-epi intermediate, (^{13}C NMR spectrum shown in figure 15) has a molecular weight preferably comprised from 1500 to 15000 Da or more preferably from 3000 to 9000 Da. The K5-OS6OSNH$_2$-epi intermediate is characterized by a free aminic group on the amino sugar, non sulphated and preferably free from formyl groups. It is also characterized by an anticoagulant activity lower than 10 IU/mg as found by measurement of the anti factor Xa activity with a chromogenic method (Coatest heparin kit, Chromogenix).The K5-OS6OSNH$_2$,epi intermediate is useful, for example, in the preparation of N-sulphated derivatives according to the invention and it does not have any anticoagulant activity.

In a particularly preferred embodiment the process for the production of the bioheparins from bacterial polysaccharides, such as the N-acetylheparosan (K5 polysaccharide of E.coli) comprises the following steps:

Preparation and purification of N-acetylheparosan polysaccharides

The starting material is preferably the N-acetyleparosan polysaccharide represented by a chain of the disaccharidic units [-4)-GicA α 1-4 GlcNAc-(1-]$_n$ constituted by a D-glucuronic acid and a N-acetyl-glucosamine monomers lonked by β 1-4 bonds. The polysaccharide can be obtained for example from natural

Escherichia coli K5 strain (strain Bi 83337/41 serotype O10:K5:H4) (in this case the polysaccharide is the K5 polysaccharide) or from Pasteurella multocida bacteria type D, or from their derivatives or mutants or Escherichia coli recombinant strains obtained for example as described in Finke A, et al. Journal of Bacteriology, 173 (13): 4088-4094, 1991, or in Drake CR, Roberts IS, Jann B, Jann K and Boulnois GJ. FEMS Microbiol Lett. 54 (1-3): 227-230, 1990.

The Escherichia coli strain useful for the production of K5 polysaccharide can be obtained also from public collections of microorganisms such as ATCC (American Type Culture Collection-USA) n° ATCC 23506.

Multocida Pasteurella type D stock can be obtained from ATCC collection (ATCC n° 12948).

The N-acetylheparosan polysaccharide is obtained by microbial fermentation and extraction from the culture broth. The purification is performed by known techniques, as those described for example in patent WO 01/02597 in which is used the following culture broth: skimmed soya flour 2g/l, K_2HPO_4 9.7 gr/l, $KH_2PO_4$2 gr/l, $MgCl_2$ 0.11 gr/l, sodium citrate 0.5 gr/l, ammonium sulphate 1gr/l, glucose (sterilized apart) 2gr/l, water q.b at 1000 ml, pH 7.3.

A pre-culture is preferably inoculated with a cell suspension of E. coli Bi 8337/41 (O1O:K5:H4) derived from a slant kept in Tripic soy agar. It is incubated at 37°C for 24 hours under stirring. In a subsequent step a fermentor containing the cited medium, is inoculated at 0,1% with the above mentioned preculture and a fermentation for 18 hours at 37°C is carried out. During fermentation the pH, oxygen, residual glucose, produced K5 polysaccharide and bacterial growth are monitored.

At the end of fermentation the temperature is brought to 80°C for 10 minutes. The cells are separated from medium through centrifugation at 10.000 rpm and the supernatant is filtered through filtration membranes with a 1000-10.000 Da cut-off to reduce the volume to about 1/5. The K5 polysaccharide is, then, precipitated by adding 4 volumes of acetone and recovered by centrifugation.

Deproteinization of the pellet is preferably carried out using a protease type II from Aspergillus Orizae in a buffer comprising NaCl 0,1 M and EDTA 0,15M at pH 8 containing SDS at 0,5% at 37°C for 90 minutes.

The solution is ultra-filtered with 10000 Da cutoff membranes and the polysaccharide is then precipitated with acetone. The purity of the polysaccharide is usually above 80% and is measured by at least one of the following analytical methods: calculation of uronic acids (carbazole method) proton and ^{13}C- NMR, UV and/or protein content.

a) N-deacetylation and N-sulphation of a N-acetyl heparosan polysaccharide from microbic source.

A quantity preferably comprised from 5 to10 g of purified K5 polysaccharide is solubilized in 200-2000 ml of 2N hydroxide sodium and left to react at 40-80°C until deacetylation is completed (i.e. 15-30 hours). The solution is led to neutrality. The solution containing the deacetylated K5 polysaccharide is maintained at 20-65°C and 10-40g of carbonate sodium are added in a single step as well as with 10-40g of a sulphating agent selected among reagents such as pyridine-sulfotrioxide adducts, trimethylamine-sulfotrioxide, etc.

The sulphating agent is added in a time of up to 12 hours. At the end of reaction, if necessary, the solution is brought to room temperature, and to a pH comprised from 7,5 to 8.

The product is purified from salts by known techniques such as, for example, by diafiltration with spiral membranes 1.000 Da (prepscale cartridge-Millipore). Retained product is reduced in volume until a 10% polysaccharide concentration is obtained. Concentrated solution, if necessary, can be exsiccated with known methods.

The N-sulphated/N-acetyl ratio is measured with 13 carbon NMR.

b) Enzymatic epimerisation by C5 glucuronil epimerase.

The C-5 epimerization step, which involves the epimerization of a part of the glucuronic acid in iduronic acid, is carried out with the C5 glucuronil epimerase enzyme (called C-5 epimerase) natural or recombinant either in solution or preferably in an immobilized form.

For this step, recombinant epimerase C5 enzyme as described in WO 98/48006 is used. Preferably, the recombinant enzyme modified as described in US n°09/732,026 and in Li et al J. Biol. Chem, vol 276, 23, (2001) 20069-20077) and contains an additional sequence at its N-terminal end.

The recombinant enzyme is preferably expressed and purified from insect cells or yeast cells preferably belonging to the genera of Saccharomyces Cerevisiae, Pichia Pastoris, Pichia methanolica, Hansenula polymorpha, Saccharomyces pombe, Kluyveromices lactis, Kluyveromices fragilis.

b.1) Immobilization of the C-5 epimerase on resins.

The recombinant enzyme can be immobilized on different inert matrices such as resins, membranes or glass beads derivatized with functional groups by known techniques such as cyanogen bromide, glutharaldeyde, carbodiimmide or by letting the enzyme react with a ionic exchange resin or by letting it absorb on membranes.

According to a preferred realization the enzyme is immobilized on commercial resins such as CNBr Sepharose 4B (Pharmacia) or on polystirenic or polymethacrylic resins (Resindion Mitsubishi) with epoxidic or diolic-CNBr activated groups.

Particularly preferred according to the invention is the enzyme immobilization on polymethacryli resin with diolic groups activated with CNBr in buffer NaHCO3 100-300 mM at 7.0-8.3 pH, preferably 7.2-7.8 pH at a temperature of 4-25°C for 12-72 hours.

According to the invention, the binding reaction of the enzyme to the inert matrix is carried out in the presence of K5 N-deacetylated N-sulphated substrate to avoid such binding to involve the enzyme active site with loss of activity.

Measure of the immobilized enzyme activity is carried out by letting recirculate, through a column containing the immobilized enzyme, the quantity of N-deacetylated N-sulphated K5 theoretically convertible by cpm of the immobilized enzyme, dissolved in buffer HEPES 25mM, KCl 0,1 M, Triton X100 0,01% and EDTA 0,15 M at 7,4 pH at 37°C for 24 hours with the flux of 0,5 ml/minute. After purification through DEAE chromatographic method and desalting on Sephadex G-10, the product is lyophilised and analysed for the iduronic acid content by proton NMR technique according to WO 96/14425.

b.2) Epimerisation with immobilised enzyme.

The C5 epimerisation reaction can be performed, for example, as described in WO 96/14425 in reaction buffer at pH 7.4 comprising preferably HEPES 0.04 or Tris

0.05 M, KCl 0.4 M, EDTA 0.06 M and triton X-100 and one or more additives such as, in particular, glycerol or polyvinylpyrrolidone.

The reaction con be performed as described in WO 01/72848 wherein a solution containing HEPES 25 mM, CaCl2 50 mM at 7,4 pH, at a temperature of 30-40 °C is used.

Particularly preferred, are the reaction conditions of temperature and buffer which allow the activity of glucoronyl C5 epimerase to be long-lasting and stable even after immobilization on a column.

20-1000 ml of an aqueous solution comprising 0,001-10 g of N-deacetylated N-sulphated K5 and EDTA 10-30 mM, preferably 15-25 mM, HEPES 25 mM, CaCl2 in concentration from 70 to 150 mM (preferably 75-100 mM) at pH 5,5-8,0, preferably pH 6,5-7,0 and thermostated at a temperature comprised from 15°C to 30°C (preferably 20-25°C) are allowed to circulate at a flux of 30-240 ml/hour for a time comprised from 1 to 24 hors in a column containing from 1,2 x 10^7 and 3 x 10^{11} equivalents of the enzyme immobilized on a thermostatic inert medium at a temperature comprised form 15°C to 30°C, preferably from 20 to 25°C.

The above temperature and buffer condition increase the enzyme stability on column for a working period longer than 3000 hours and, therefore, make the process particularly advantageous. At the end of reaction the sample is purified by passing through a DEAE resin or a Sartobind DEAE cartridge and precipitated by addition of 2M of NaCl and, finally desalted on G10 Sephadex resin (Pharmacia) or purified through precipitation with 2 volumes of ethanol and passing on IR 120H+ resin to obtain the sodium salt.

The product obtained under said preferred conditions has an epimerisation rate measured by proton NMR technique as described under WO 96/14425, of at least 50% (iduronic acid rate on total uronic acids).

Controlled depolymerization.

The product obtained under step b) or c) or d) undergoes controlled depolymerization with known techniques such as deamination with nitrous acid as described in WO 82/03627, or by an oxidative opening with sodium periodate (EP 287477), or by free radicals treatment (EP 121067) or by beta-elimination (EP40144), or by gamma rays treatment (US 4,987,222) to obtain molecular

weight fractions preferably comprised from 1500 to 15000 D, or even more preferably comprised from 3000 to 9000 Da.

According to a preferred aspect of the invention, controlled depolymerization is carried out before the sulphation steps.

In particular, the product resulting from the former steps is put under controlled depolymerisation with nitrous acid or with sodium nitrite. In this case the salt quantity used is comprised from 1 to 100 mg for each gram of polysaccharide, followed by reduction by borohydride in excess.

According to a second preferred embodiment, the sample is dissolved in 50-250 ml of water at 4°C ad acidified with 1N chloride acid. A sodium nitrite quantity comprised from 5 to 500 mg is then added and the reaction is continued for less than 60', preferably less than 30'.

After destruction of the sodium borohydride in excess, the product is recovered by precipitation with 3 ethanol volumes and exsiccated in a vacuum oven.

When depolymerization is carried out at the end of the process, it can be performed as described for example in WO 01/72848.

c) Partial O-sulphation combined with partial O-desulphation.

The product derived from the steps above is re-suspended in water at a concentration of 10%. The solution is cooled down at 10°C and passed while keeping the temperature at 10°C through a IR-120 H^+ cationic exchange resin. After the solution is passed through the resin is washed with deionised water until the eluate pH is higher than 6. The acidic solution is led to neutrality by adding a tertiary ammine or a quaternary ammonium salt such as a 15% hydroxide tetrabutylammonium aqueous solution obtaining the relevant ammonium salt. The solution can be concentrated to a minimum volume and lyophilised.

The product obtained is suspended in 10-1000 ml of an organic solvent consisting, preferably of sulfolane or 2,4-dimethylsulfolane. Alternatively, the organic solvent can be dimethylformamide (DMF) or dimethylsulfoxide (DMSO) or N,N,-dimethylacetamide. This organic solvent is added with a sulphating agent such as a pyridine adduct $-SO_3$ in solid form or in solution with the same solvents previously used.

The molar ratio between the sulphating agent and the polysaccharidic substrate (epimerised N-sulphated K5), to be intended as the ratio between the sulphating agent and the hydroxyl moles of the polysaccharidic dimer, is maintained equal to or lower than 5 or, more preferably, lower than 2,5 or, even more preferably lower than 1,5.

The solution is maintained at a temperature comprised from 20 to 70°C, preferably comprised from 30 to 60°C for a period of time lower than or equal to 10 hours, more preferably equal to or lower than 8 hours, or even more preferably, equal to or lower than 6 hours. At the end of reaction the solution is eventually cooled down at room temperature and added with acetone saturated with chloride sodium till complete precipitation of the polysaccharide.

The precipitated is separated from the solvent by filtration, solubilized with the minimum quantity of deionised water and added with chloride sodium until a 0,2 M solution is obtained. The solution is brought to pH 7,5-8 by sodium 2 N hydroxide addition. Acetone is then added to complete precipitation. The precipitated is separated from solvent by filtration. The obtained solid is solubilized with 10-100 ml of deionised water and purified from residual salts through ultrafiltration.

An aliquot is lyophilised for the structural analysis of the partially O-sulphated product by ^{13}C-NMR and ^{1}H-NMR.

The solution containing the partial sulphated product is passed through a IR-120^{+} cationic exchange resin or equivalent.

The resin is washed with deionised water until the pH of the eluate is higher than 6 and is then added with pyridine. The solution is concentrated to a minimum volume and lyophilised. The product is treated with 20-200 ml of a solution of DMSO/methanol (9/1 V/V) and the solution is kept at 45-90°C for 10-420 minutes.

At the end the solution is added with 10-200 ml of deionised water and treated with acetone saturated with sodium chloride to complete precipitation.

The solid obtained is purified through diafiltration according to known techniques and an aliquot is lyophilised for structural analysis by ^{13}C-NMR.

d) partial 6-O sulphation

The product from the previous step is then 6O-sulphated. The content of sulphate groups in 6O position is measured by known techniques such as for example NMR

according to the conditions described in Guerrini et al. Seminars in Thrombosis and Hemostasis, vol 27, 5, 473-482, 2001.

The product obtained is resuspended in water at a concentration comprised from 5 to 10% and kept at room temperature. The solution is then passed through a resin having IR-120 H^+ cationic exchange or equivalent. After the solution flow, the resin is washed with deionised water and led to neutrality with a tertiary ammine or a quaternary ammonium salt such as, for example, hydroxide tetrabutilammonium in aqueous solution obtaining the relevant ammonium salt. The solution is concentrated to a minimum volume and liophylized.

The product obtained is suspended in 10-1000 ml of an organic solvent consisting dimethylformamide preferably, in sulfolane or in 2,4-dimetilsulfolane or in N,N,-dimetylacetamide and added with a sulphating agent such as $-SO_3$ pyridine adduct in solid form or in solution with the same solvent.

The suspension is brought to a temperature comprised from 4°C to 30°C, preferably from 10 to 25°C, treated with quantity of a sulphating agent such as the pyridine-SO_3 adduct using less than 2 equivalents compared to the hydroxile groups to be sulphated or even more preferably with less than 1.5 equivalents for 10-90 minutes and treated with acetone saturated by sodium chloride in such a quantity to complete precipitation. The solid obtained solid is then purified by diafiltration according to methods well known in the art. An aliquot is lyophilised for the structural analysis by ^{13}C-NMR.

e) N-risulphation

The solution from step d) containing the 6O sulphated polysaccharide, is brought to 20-65°C and added with 10-100g of carbonate sodium with a single addition and with 10-100g of a sulphating agent selected among available reagents such as, preferably, the pyridine-sulfotrioxide. The addition of the sulphating agent is carried out during a variable time up to 12 hours. At the end of the reaction, if necessary, the solution is brought to room temperature, and to a pH comprised from 7,5 to 8, preferably with 2M sodium hydroxide.

The product is purified from salts by known techniques such as, for example, dialfiltration using a spiral membrane of 1000 Da (i.e. Prepscale cartridge-Millipore). The process is complete when the permeate conductibility is below

1000 µS, preferably below 100 µS. The product obtained is reduced in volume till a polysaccharide concentration of 10% is obtained by filtration. An aliquot of the concentrated solution is lyophilised for structural analysis by ^{13}C-NMR.

The 6-O selective sulphation and N-risulphation carried out as described in this step, on products derived from step e) can be performed in a different order for example the N-risulphation at first and then the 6-O sulphation) as described in WO 98/42754, without modifying the biological activities of the final product.

Enrichment in sequences binding the III antithrombin (optional).

The product obtained as described in the previous step can be optionally further purified by chromatography for example on an anionic exchange chromatographic column such as for example on DEAE columns as described in Lam L.H. et al. Bioch. And Biophysical Research Communication vol 69, 2, pag. 570-577, 1976. Alternatively to this purification or besides this, the product can be subjected to a further affinity chromatography on columns carrying the whole or the partial human antithrombine sequence as described, for example, in Hook et al FEBS Lett 1976, 66:90-93 or in US patent 4,692,435 or peptide sequences having a high affinity for heparin as described in Liu S et al. Proc. Natl. Acad Sci USA 1980, 77:6551-6555. This treatment allows the separation, by subsequent elution with a NaCl saline solution of at least a fraction able to bind to the solid phase.

In practice, 10-50 mg of product derived form the N-risulphation step are loaded on an affinity column where 50-100 mg of human antithrobin III are immobilized (Kedrion SpA, Lucca, Italia) in buffer Tris-HCl 10 mM at 7.4 pH and 0-0.15 M NaCl 4°C. The column is then washed with at least 3 volumes of buffer Tris-HCl 10 mM 7.4 pH.

The molecules bound with higher affinity to the column are eluted by Tris HCl 10 mM 7.4 pH containing from 0.5 to 3 M of NaCl.

The eluted material is, then, preferably diafiltrated by 1000 Da cut-off spiral membranes to eliminate salts and concentrated by lyophilization.

An aliquot can be analyzed by the Carbazole method, HPLC, NMR and chromogenic test to measure the antiXa activity.

The obtained material shows a greater antiXa activity enriched from 1.5 to 3 times the activity of the starting material.

The same increase in anti-Xa activity is obtained by treating the same way extractive heparins. This is a further signal of the likeness of the biotechnological heparins obtained according to this invention to extractive heparins, in binding to antithrombin III.

EXPERIMENTAL PART

EXAMPLE 1. Biotechnological heparin production according to the invention process.

The following steps have been followed:

a) N-acetylheparosan polysaccharide preparation starting from Escherichia coli K5

b) N-deacetylation/N-sulphation

c) Epimerization

d) Depolymerization

e) partial O-sulphation/partial O-desulphation

f) partial 6-O sulphation

g) N-risulphation

a) Polysaccharide preparation

N-acetylheparosan polysaccharide was obtained through fermentation of E.coli Bi 8337/41 stock, serotype O10:k5:H4 (ATCC 23506) and subsequent extraction from culture broth and purification according to the description in patent WO 01/02597 using the following culture broth: skimmed soy flour 2 gr/l, K_2HPO_4 9.7 gr/l, K_2HPO_4, 2 gr/l M_gCl_2 0.11 gr/l, citrate sodium 0.5 gr/l, sulphate Ammonium 1 gr/l, Glucose (sterilized aside) 2 gr/l, water q.b at 1000 ml, pH 7.3.

The culture was inoculated with a cell suspension derived from a slant kept in Triptic soy agar at 37°C for 24 hours under stirring. The inoculum in fermentor, type F5 (industrie Meccaniche di Bagnolo SpA) containing the same abovementioned medium, was inoculated at 0,1% with the abovementioned beuta culture and the fermentation was performed at the temperature of 37°C for 18 hours. During fermentation were measured the pH, oxygen, residual glucose, produced K5 polysaccharide and bacterial growth. At the fermentation end, the temperature was taken to 80°C for 10 minutes. The cells were separated from medium through centrifugation at 10.000 rpm and the supernatant filtered using filtration membranes with 10.000 Da cut-off to reduce volume to 1/5. The K5

polysaccharide was precipitated by adding 4 volumes of acetone and, finally, recovered by centrifugation.

Deproteinization of the solid obtained was performed by protease type II from Aspergillus Orizae in buffer of NaCl 0,1 M and EDTA 0,15 M at pH 8 containing 0,5% SDS, 37°C for 90 minutes.

The solution obtained was ultrafiltered with membranes having a nominal cut-off of 10.000 Da and the polysaccharide was precipitated with acetone. The polysaccharide purity was measured by uronic acids determination (Carbazole method), proton and carbon 13 NMR, UV and protein content.

b) N-deacetylation/N-sulphation

10 gr of the product obtained from step a) was solubilized in 200 ml of 2N hydroxide sodium and left at 50°C for 18 hours. The solution was brought to neutral pH with 6N hydrochloride acid. A N-deacetylated polysaccharide was obtained.

The N-deacetylated polysaccharide solution was kept at 40°C and added with 10 g of carbonate sodium in a single addition and 10 g of pyridine-sulphotrioxide adduct in 10 minutes. The product obtained made up of N-deacetylated N-sulphated K5 polysaccharide, was purified from salts by diafiltration using a spiral membrane of 1.000 D (prepscale cartridge-Millipore). The purification process was completed when the permeate conductiveness was below 100 µS.

The product was brought to a 10% polysaccharide concentration using the same diafiltration method and was then lyophilized.

The N-sulphate/N-acetyl ratio of the obtained product was 9,5/0,5 as measured through carbon 13 NMR.

c) Epimerization

c-1) C5 Epimerase immobilization on resin.

5 mg of recombinant glucuronyl C-5 epimerase obtained according to US n° 09/732,026 and to Li et al J. Biol. Chem., vol 276, 23, (2001) 20069-20077, were dissolved in 200 ml of buffer Hepes 0,25 M, 7,4 pH, containing KCl 0,1 M, Triton X-100 0,1% and EDTA 15mM, 100 mg of N-deacetylated N-sulphated K5 obtained according to the description in step b) were added to the solution. The solution was diafiltrated in a 30.000 D membrane at 4°C till N-deacetylated N-sulphated K5

disappeared in diafiltrated. To the solution retained by the membrane the buffer was changed by diafiltration and by substituted with $NaHCO_3$ 200 mM at 7 pH and after concentration to 50 ml we added 50 ml of CNBr Sepharose 4B activated resin, which was left to react overnight at 4°C.

At the end of reaction the residual enzyme quantity in supernatant was measured with the Quantigold method (Diversified Biotec) after decantation. The enzyme in supernatant was absent, demonstrating that, with the method described, the enzyme was immobilized at 100%. To occupy the resin sites left available, the resin was washed with tampon Tris-HCl 100 mM at 8 pH.

For the measurement of the immobilized enzyme activity, a quantity of the immobilized enzyme theoretically corresponding to $1,2 \times 10^7$ cpm, was loaded in a column. In the column so prepared, 1 mg of N-deacetylated N-sulpahted K5 obtained according to step b) was treated and dissolved in buffer HEPES 25 mM, KCl 0,1M, EDTA 0,015M, Triton X-100 0,01% at 7,4 pH, allowing it to recirculate through said column at 37°C overnight with a flux of 0.5 ml/minute.

After purification by DEAE chromatography method and desalification on Sephadex G10, the sample was lyophilized and analyzed for the iduronic acid content through proton NMR technique according to the description on patent WO 96/14425.

c-2) Epimerization with immobilized enzyme.

10 g of N-deacetylated N-sulphated K5 polysaccharide were dissolved in 600 ml of EDTA 15 mM buffer, HEPES 25 mM, 7,0 pH, containing $CaCL_2$ 75 mM. The obtained solution was allowed to recirculate through a 50 ml column loaded with a resin containing the immobilized enzyme.

This operation was performed at 28°C with a flux of 200 ml/h for 24 hours.

The product obtained was purified by ultrafiltration and precipitated with ethanol. The precipitate was resolubilized in water at a concentration of 10%.

The obtained product shows an Epimerization percentage, measured with ^1H-NMR, as iduronic acid percentage on the total uronic acids of 55% (as shown in picture 1).

d) Controlled Depolymerization.

The sample obtained in step c-2) underwent to controlled degradation with nitrose acid as described in patent WO 82/03627, In particular, 5 g of the sample were dissolved in 250 ml of water and taken to 4°C with thermostated bath. The pH brought to pH 2.0 with 1 N chloride acid cooled at 4°C and, afterwards, 200 mg of nitrite sodium were added. When necessary the pH was brought to 2 with 1 N chloride and kept under slow stirring for 15 minutes. The solution was neutralized with NaOH 1N cooled down at 4°C.

We added 250 mg of sodium boro-hydride dissolved in 13 ml of deionized water leaving to react for 4 hours. The solution was taken to 5,0 pH with 1N chloride acid and left for 10 minutes to destroy the excess of sodium borohydride, and, afterwards, it was neutralized with NaOH 1N. The product was recovered through precipitation with 3 volumes of ethanol and, then, exsiccated in a vacuum stove. The obtained product shows a molecular weight of about 6000 Da.

e) O-partial sulfation / O-partial desulfation

The product resulting from the previous step was re-suspended at a 10% concentration in water solution. The solution was cooled at 10°C and allowed to flow at the temperature of 10°, through a cationic exchange resin IR-120$^+$. After the flow of this solution, the resin was washed with deionized water, till the pH of the eluate was higher than 6. The acid solution was then led to neutrality by using a tertiary amine or a quaternary ammonium salt, such as for example tetrabutyl ammonium hydroxide in aqueous solution at 15%, obtaining as a result the ammonium salt. The solution was then concentrated at a minimum volume and lyophilized.

The resulting solution was resuspended into 100 ml of N,N, dimethylacetamide (DMA) and pyridine –SO_3 was added.

Then a quantity of sulfating agent in a molar ratio between the sulfating agent and the epimerised K5 N-sulfate substrate (as hydroxyl moles) of 1,25 was added.

The solution was kept at 50°C for 360 minutes. At the end of the reaction the solution was cooled at room temperature and added with acetone saturated with sodium chloride till complete precipitation.

The precipitated was separated from the solvent by filtration, solubilized with a minimum quantity of deionized water and added with sodium chloride till a

solution of 0.2 M was obtained. The solution was brought to pH 7,5 by addition of sodium hydroxide 2 N and acetone was added to allow precipitation. The precipitated solution was then separated from solvent through filtration. The solid solution thus obtained was solubilized by addition means of 100 ml deionised water and purified from residual salts by ultra filtration.

An aliquot was lyophilized for the structural analysis of the partially O-sulfated product by ^{13}C-NMR.

The solution containing the partially sulfated product was allowed to flow through a cation exchange IR-120 H^+ resin or equivalent. After the flow of this solution, the resin was washed with deionized water until the permeated pH was higher than 6. The acid solution was led to neutrality by adding pyridine. The solution was concentrated at a minimum volume and lyophilized. The product obtained was handled with 100 ml DMSO/methanol (9/1 V/V) solution and the obtained solution was kept at 65°C for 240 minutes.

Finally, the solution was added with 200 ml deionised water and then handled with acetone sodium chloride saturation in such a quantity to complete the precipitation. The solid obtained was purified through diafiltration according to known techniques and an aliquot was lyophilized for structural analysis by ^{13}C-NMR.

f) partial 6-O sulphation

The product obtained from step e) was then re-suspended into a water solution at a concentration of 10% and kept at room temperature. The solution was passed through a cationic exchange resin IR-120 H^+. The resin was then washed with deionised water and led to neutrality by means of hydroxide tetrabutylammonium in aqueous solution, obtaining the ammonium salt. The solution was then concentrated in minimum volume and lyophilized.

The product obtained was then suspended into 100 ml of DMF and the sulfating agent pyridine -SO3 added into a DMA solution was then added. The solution brought to 10°C and treated with a quantity of as sulfating agent pyridine -SO3 adduct with respect with 1,25 equivalents of sulphating agent in respect to hydroxyl for 60 minutes.

The solution was treated with acetone saturated with sodium chloride in a quantity to complete precipitation. The solid obtained was purified through diafiltration according to known method. A aliquot was lyophilized for the structural analysis by ^{13}C-NMR.

g) N-risulphation

The product was solubilized in water, brought to a temperature of 40°C and added by a single addition of 10 gr sodium carbonate and 10 gr pyridine -sulfotrioxide in a time of 10 minutes.

At the end of the reaction, when necessary, the solution was led to room temperature, and then, if necessary, at pH lower than 8.0 with NaOH.

The product was then purified from salts by known techniques, such as for example by diafiltration with a 1000 Da cut-off spiral membrane (prep scale cartridge-Millipore). The process ended with a permeate conductivity lower than 1000μS, preferably lower than 100 μS. The retained product was reduced in volume till we obtained a 10% concentration of the polysaccharide by the same filtration process was achieved.

The spectrum 1H-NMR is shown in picture 2.

The anti-Xa activity measured into human plasma of the obtained products was 140 IU/mg (see table 2) and the ratio between the anti-Xa activity and the anti-II activity was 2.5.

EXAMPLE 2. O-sulphation with DMF

Example no. 1 was repeated with the variation that in step c) and f) the partial O-sulphation and partial 6O-sulphation were carried out using dimethylformamide (DMF) as organic solvent. The obtained product showed an anti-Xa activity in plasma of 85.9 IU/mg (see table 2). NMR spectrum is shown in figure 3.

EXAMPLE 3. Controlled Depolymerization in presence of 50 mg/gr substrate of sodium nitrite

Example no.1 was repeated with the difference that the controlled depolymerization was performed with 50 mg sodium nitrite per g of polysaccharide in order to obtain a molecular weight of about 4200 Da.

The product obtained showed an anti-Xa activity in plasma of 60.1 IU/mg (see table 2). The ^{13}C-NMR spectrum is shown in figure 4.

EXAMPLE 4. Production of biotechnological heparins having a molecular weight of about 8000Da (controlled depolymerization with 20 mg/g substrate of sodium nitrite)

Example no.1 was repeated with the difference that in step d) the controlled depolymerization was carried out with 20 mg sodium nitrite per g of polysaccharide in order to obtain a molecular weight of about 8000Da.

The obtained product showed an anti-Xa activity in plasma of 150 IU/mg (see table 2). The ^{13}C-NMR spectrum is shown in figure 5.

EXAMPLE 5. Biotechnological heparins production having molecular weight of around 20000 Da

Example no. 5 was performed according to the following steps:

a) preparation of N-acetylparosane polysaccharide starting from Escherichia coli K5;

b) N-deacetylation/N-sulphation;

c) epimerization;

d) partial O-Sulphation/partial O-desulphation;

e) partial 6-O sulphation/N-risulphation,

Steps a)-c) correspond to their respective steps in example 1, and where, lacking the depolymerization (step d), step d) corresponds to step e) in example 1 and step e) corresponds to step f) and g) in example 1.

The final product obtained had a molecular weight of about 20.000 Da, and is susceptible of depolymerization. The anti-Xa activity in plasma was 135 IU/mg (see table 2).

The ^{13}C- NMR spectrum is shown in figure 6.

EXAMPLE 6. Production of biotechnological heparins production having molecular weight of around 15000 Da (controlled Depolymerization with 5 mg/g substrate of sodium nitrite).

Example 1 was repeated under the same temperature and time conditions used in step d) but the controlled depolymerization with sodium nitrite was carried out with 5 mg of sodium nitrite per g of polysaccharide in order to obtain a molecular weight of about 15000 Da.

The obtained product showed an anti-Xa in plasma activity of 180 IU/mg (see table 2); the ^{13}C-NMR spectrum is shown in figure 7.

EXAMPLE 7. Process of preparation of biotechnological heparin according to known techniques.

In this example the O-super sulphation and 6O-sulphation process conditions described in WO 01/72848 and WO 02/50125 were used. Briefly, performing the supersulphation in dimethylformamide at 50°C for 18 hours, the O-desulphation at 65°C for 150 minutes and the 6O-sulphation in dimethylformamide at 0°C for 90 minutes.

The final product showed a molecular weight of about 20.000 Da and an anti-Xa activity in plasma of 45 IU/mg (see table 2). The ^{13}C-NMR spectrum is shown in figure 8.

EXAMPLE 8. Production of biotechnological heparins according to prior art techniques.

This example was performed by using the same supersulphation and 6O-desulphation conditionsdescribed in WO 01/72848 and WO 02/50125: in brief, the supersulphation was performed in dimethylformamide at 50°C for 18 hours, the O-desulphation at 65°C for 150 minutes and the 6O-sulphation in dimethylformamide at 0°C for 90 minutes.

Moreover, a depolymerization step was performed at the end of sulphation process as described in WO 01/72848 and WO 02/50125 in the presence of 40 mg/g substrate of sodium nitrite at 4°C for 15 minutes.

The obtained final product has a molecular weight of about 6.000 Da and an anti-Xa activity in plasma of 31.5 IU/mg. The ^{13}C-NMR spectrum of is shown in figure 9.

EXAMPLE 9. Production of biotechnological heparins: control of depolymerization and sulphation conditions.

Example 1 was repeated with the following variations:

The controlled depolymerization (step d) in example 1) was performed with 40 mg of sodium nitrite per g of epimerised K5NS under the same time and temperature conditions of example 1. The molecular weight obtained was about 6500 Da.

Partial sulphation (step e) of example 1 was performed with a molar ratio of 5 between sulfating agent and substrate K5N-sulfated epimerised and for a period of 180 minutes, while O-desulphation was made for 60 minutes.

The product obtained showed an anti-Xa activity in plasma of 89 IU/mg (see table 2). The ^{13}C-NMR spectrum is shown in figure 10.

EXAMPLE 10. Production of biotechnological heparins: control of the depolymerization and sulphation conditions.

Example 1 was repeated and the intermediate depolymerization (step d) on K5-N polysaccharide was performing under the same time and temperature conditions described in Example 9 but using 40 mg of sodium nitrite per mg of epimerised K5NS to obtain a molecular weight of about 6500 Da. The partial sulphation corresponding to (step e) in example 1) was carried using molar ratio of 0.6 between sulfating agent and the epimerised substrate K5N-sulfated in a 8 hours incubation time. O-desulphation was carried out for 30 minutes.

The product obtained showed an anti-Xa activity in plasma of 101 IU/mg (see table 2).

^{13}C-NMR spectrum of the product obtained according to this example is shown in figure 11.

EXAMPLE 11. Separation of polysaccharide fractions having great affinity for antithrombin III by means of selection on affinity column.

A polysaccharide was prepared according to Example 1. After step g) of N-risolphation the product was allowed to pass through an affinity column as follows:

20 mg of product obtained from step g) in example 1 were loaded on a CNBr sepharose 4B resin column (Pharmacia), on which were previously immobilized, according known techniques, 100 mg of human antithrombin III (Kedrion SpA, Lucca, Italia), in buffer Tris-HCl 10 mM at pH 7.4 and 0-0.15 M NaCl, at 4°C. After a period of 60 minutes of binding, the column was washed with at least 3 volumes of buffer Tris-HCl 10 mM pH 7.4.

The molecules bound with a greater affinity to the column were eluted by adding a gradient of Tris HCl 10 mM pH 7.4 containing 2M NaCl.

The eluted material was diafiltrated by a 1000 Da cut-off spiral membrane to eliminate the salts and concentrated by lyophilization.

The final product showed a molecular weight of 8500 Da and an anti-Xa activity in plasma of 300 IU/mg 8see table 2). ^{13}C-NRM spectrum of is shown in picture 12.

EXAMPLE 12. biotechnological heparins production having low molecular weight of about 6000 Da.

Example 5 was repeated but after the N-resulphation step the product was depolymerised under the same conditions as described in step d) of example 1.

The final product obtained had a molecular weight of about 6.000 Da and an anti Xa activity in plasma of 65 IU/mg (see table 2). The ^{13}C-NMR spectrum is shown in figure 13.

EXAMPLE 13. Biotechnological heparins production: control of the sulphation conditions.

Example 1 was repeated varying the O-sulphation (step e) of Example 1, which was carried out with a molar ratio of 5 between the sulphating agent and the epimerised K5-sulphated substrate) for 8 hours at 50°C.

The product obtained showed an antiXa activity in plasma of 75 IU/mg. The ^{13}C-NMR spectrum is shown in figure 14.

EXAMPLE 14. Preparation of K5-OS6OSNH$_2$,epi intermediate (non re-sulphated intermediate of biotechnological heparin).

Example 1 was repeated without the last step of resulphation.

The final product had a molecular weight of about 6.000 Da and an anti Xa activity of 5 IU/mg (see table 2). The ^{13}C-NMR spectrum is shown in figure 15.

EXAMPLE 15. Preparation of K5-OS,NH2,epi intermediate (intermediate of biotechnological heparin before the desulpahtion, 6O-sulphation and N-resulphation steps).

Example 1 was repeated by varying the partial O-sulphation (step e) of Example 1) conditions, as it was carried out with a molar ratio of 5 between sulphating agent and the hydroxils of the epimerised K5N-sulphate substrate for 8 hours at 50°C and without the subsequent steps of desulphation, partial 6O-sulphation and N-resulphation.

The obtained K5-OSNH$_2$-epi product had a molecular weight of about 6.000 Da, a 95% hydroxils sulphation and an anti Xa activity of 8 IU/mg (see table 2). The proton ^{1}H-NMR spectrum is shown in figure 16.

EXAMPLE 16. Production of biotechnological heparins with molecular weight about 6.000 Da.

Example 1 was carried out with the following steps:

a) preparation of N-acetylheparosan polysaccharide starting from K5 Escherichia coli

b) N-deacetylation/N-sulphation

c) epimerization

d) partial O-sulphation/partial O-desulphation

e) controlled depolymerization

f) partial 6-O sulphation/N-resulphation,

where the conditions of steps a)-c) correspond to those described in the respective steps of example 1 and where steps d) and e) are in an inverted order with respect to the same steps of example 1.

The obtained final product had a molecular weight of about 6.000 Da, and an anti Xa activity in plasma of 95 IU/mg (see table 2). The carbonium NMR spectrum is showed in picture 17.

EXAMPLE 17. Determination of the biological activity of the products obtained according to invention.

Measurement of the anti factor Xa activity: The anti factor-Xa activity was extimated according to a chromogenic method (Coatest Heparin kit, Chromogenix). The measure was performed in normal human plasma, using as reagents the chromogenic substrate S2222 (Chromogenix), Xa bovine factor (Chromogenix) and human Antithrombin III (Chromogenix). The reaction was performed at 37°C in a coagulometre ACL 9000 (International Laboratory) and the reading was made at 405 nm. Results are shown in table 2. Results obtained according the known techniques (particularly products of the procedures made as described in WO 01/72848 and WO 02/50125) have been summarized in table 1, where data of the literature for extractive heparin have been reported (Fareed J et al. Exp Opin. Invest. Drugs, 1997, 6:705-733).

Anti-factor activity IIa. Anti-factor activity IIa was extimated in normal human plasma according to the following protocol:

30 µl of 0.5 U/ml of human antithrombin III (Chromogenix) were mixed to 30 µl of a solution of the sample to be investigated in different concentration and to 60 µl of bovine thrombin to at 5.3 nKat/ml (Chromogenix).

The solution was incubated for 70 seconds at 37°C, then 60 µl of chromogenic substrate S-2238 (Chromogenix) were added. The reaction was recorded for 90 seconds with a reading every second at 405 nm using a coagulometre ACL 9000 (International Laboratory). The results obtained with the known products (mainly products of the process described in WO 01/72848 and WO 02/50125) have been summarized in table 1.

Resistance to heparinase. The resistance to heparinase I was estimated by preparing low and a high molecular weight samples, in buffer Tris HCl 20 mM, 50 mM NaCl, 4mM CaCl2 and 0.01% BSA at pH 7.5 at a final concentration of 0.02%. 20 units of heparinase I (Sigma, CAS number 52227-76-6) were added to a 100 µl solution containing the sample and the reaction was incubated at 25°C. The reaction was then stopped by addition of HCl 50 mM, at regular intervals every 10 min, 1 hour, 2 hours, 20 hours.

Every sample was analyzed both by spectrophotometrical determination at 235 nm and by GPC-HPLC to determine the molecular weight. The results obtained are shown in table 3, where it can be observed that while extractive heparins used in the study both high (HMW) and low molecular weight (Fraxiparina, Sanofi) have been degraded in the long run as shown by the decrease in the molecular weight, biotechnological heparins obtained according to this invention are stable (molecular weight is stable even after 20 hour treatment with heparinase I).

TFPI activity. The determination of the activity on the basis of TFPI factor was made in vitro on HUVEC cells according to the method described in Gori AM. et al. Thromb. Haemostasis 1999:81:589-93 and the comparison was made with a commercial non-separated heparin (table 4).

Inhibition of proteases generation. The determination of inhibition of the proteasis generation was performed in plasma depleted of fibrinogen according to the method reported in Fareed et al. Path. Haem. Thromb. 2002; 32 (3): 56-65. After adding various dilutions of the samples under examination, plasma was activated

by adding PT (thromboplastine C) for the activation of the intrinsic coagulation system or APTT (Dade Actin) for the activation of the intrinsic coagulation system. The inhibition of both thrombin (factor II) and of Xa factor was monitored using a ACL 9000 coagulometer (International Laboratory). The values obtained with a sample dilution of 50 µg/ml are shown in table 5.

Affinity to factor PF4: determination of the affinity to factor PF4 (platelet factor 4) was evaluated in plasma by determining the residual anti Xa activity after addition of a fixed quantity of PF4 factor in the solution containing the biotechnological heparins obtained according to the invention.

100 µg plasma containing 0.8 anti Xa IU/ml were added obtaining a PF4 final concentration of 10 µg/ml. The residual anti Xa activity of the sample was measured using a Coatest heparin kit (chromogenix) by the coagulometer ACL 9000 (International Laboratory). The residual anti Xa activity was measured as percent of the initial activity (table 6).

Measurement of activated partial thromoplastine time (APTT): the determination of APTT was performed by coagulation test using a coagulometer ACL 9000 (International Laboratory). The reaction was carried out at 37°C by adding a quantity of cephalin (kit APTT International Laboratory cod 8468710) to the sample duly diluted and following the clot formation after calcium chloride addition and the reading the out put at 660 nm.

The APTT values are reported in table 2 as percent of activity with respect to the first international standard of low molecular weight heparin 85/600.

Determination of the activity of heparinic cofactor II (HCII): the determination of HCII was carried out by preparing a reaction mixture containing 20 µl HCII (Stago) 0.085 PEU/ml, 80 µl solution of the sample under examination at different concentration, 50 µl of thrombin 0.18 U/ml (Boehringer) in 0.02 M tris-buffer, pH 7.4, =.15 M NaCl and 0,1% of PEG 6000. The solution was incubated for 60 seconds at 37°C, then, 50 µl of 1 mM of Spectrozyme chromogenic substrate (American Diagnostic) were added. The reaction was monitored for 180 seconds at 1 seconds intervals at 405 nm wave-length on an automatic coagulometer ACL 9000 (instrumentation Laboratory).

HCII values are reported in table 2 as percent of activity with respect to the first international standard of low molecular weight heparin 85/600.

In table 1 and 2 the biological activity data of biotechnological heparins produced according to the invention, or according to methods characterized by O-supersulfation regardless to the use of solvents donor or non donor of formyl groups such as N,N, dimethylformamide and by a final depolymerization step, or of extractive heparins. The data show in particular that the anti-Xa activity measured in human plasma is higher for the products obtained according to this process than for the prior art products at comparable molecular weight.

In fact in products obtained according to the invention the ratio between biological activity expressed as anti factor X activity and the molecular weight is higher. Such an increase seems to be due to the combined peculiarities of the process:

- O-sulphations (O-sulphation and 6O-sulphation) performed in mild conditions (see examples 5 and 7 for a comparison);
- use of an aprotic polar solvent non donors of formyl groups during the O-sulphation and the 6O-sulphation steps (see examples 1 and 2 for a comparison);
- intermediate depolymerization step, which is performed before the O-sulphation or before the 6O-sulphation steps, compared to depolymerizaion carried out at the end of the process (see examples 1 and 12 for a comparison).

A higher increase of the ratio between the biological activity, considered as the anti Xa activity in plasma and the molecular weight of the product obtained, as well as of the ratio between the anti Xa and anti IIa activity is obtained when the O-sulphations (O-sulphation and 6O-sulphation) are partial together with the use of a polar aprotic solvent non donor of formyl groups for O-sulphation and 6O-sulphation coupled to depolymerization performed in an intermediate phase as in example 1 and in example 6.

However, the process is compatible also with a stronger O-sulphation combined with partial 6O-sulphation, as described in example 13.

Moreover, the process of the invention is also compatible with a final depolymerization as described in example 12.

An additional parameter improved in the products of the invention, as compared to biotechnological heparins obtained according to known method, is the ratio

between anti-Xa activity and anti IIa activity indicating a relation between antithrombotic and anticoagulant characteristics, resulting also from APTT values which is similar to value of extractive heparins.

In the products obtained according to the invention such ratio is equal to or higher than 1, while is lower than 1 in products obtained according to prior art processes.

Another characteristic shown by the high HCII values, is a greater ability to inhibit directly thrombin, as compared to extractive heparins.

EXAMPLE 18. Production of biotechnological heparins with molecular weight about 6.000 Da.

Example 1 was carried out according to the following steps:

a) preparation of N-acetylheparosan polysaccharide starting from K5 Escherichia coli

b) N-deacetylation/N-sulphation

c) epimerization

d) partial O-sulphation/partial O-desulphation

e) N-risulphation

f) controlled depolymerization

g) partial 6O-sulphation

where the conditions of steps a)-c) correspond to those described in the respective steps of example 1 and where steps but the d)-g) which are in an inverted order with respect to the same steps of example 1.

The obtained final product had a molecular weight of about 6.000 Da, and an anti Xa activity in plasma of 92 IU/mg (see table 2). The proton NMR spectrum is showed in figure 18.

TABLE 1. Comparative table of biological activity data.

Product	O-sulphation and depolymerization	Molecular weight range (Da)	AntiXa activity in human plasma	Anti IIa activity in human plasma	anti Xa/ MW(KDa) ratio	antiXa/anti IIa ratio
Unfractionated extractive heparin (UFH)	-	11000-15000	160-200*	160-200 *	13-14	1.0
Extractive heparin LMW	-	3900-6700	80-150*	25-60*	15-25	1.8-4.0
Product Ex. n° 7, WO 01/72848,	- Supersulphation	20000-30000	30-80	70-80	1.5-3	0.5-0.8
Product Ex. n° 8, WO 01/72848,	- Supersulphation - Final depolymerization	4000-8000	15-50	40-70	3-6	0.5-0.8
Product Ex. n° 6	- Partial O-sulphation - Partial 6O-sulphation - Intermediate depolymerization	9000-15000	150-190	100-150	10-12	1.0-1.5
Product Ex. n° 1	- Partial O-sulphation - Partial 6O-sulphation - Intermediate depolymerization	3000-9000	50-150	30-100	16-25	≥ 1.5
Product Ex. n° 11	- Partial O-sulphation - Partial 6O-sulphation - Intermediate depolymerization - Affinity column	4000-9000	100-350	50-100	25-40	≥ 1.5

(*) values published in: Fareed et al. Exp. Opin. Invest.Drugs (1997) 6:705-733, Eriksson B. et al. Tromb. Haemost. (1995) 73: 398

TABLE 2. Summary table: examples and biological activity of the products according to the invention.

Ex. n°	Figure n°	MW (Da) HPLC	O-Sulphation	desulphation	Anti Xa in plasma (IU/mg)	antiXa/ anti IIa	aPTT	HCII	notes
1	1,2	6000	r.m=1.25 6 hrs, 50°C	240 min 65°C	140	2.5	93	n.d	see example 1
2	3	6000	r.m=1.25 6 hrs, 50°C	240 min 65°C	85,9	1.5	84	364	As in example 1 but with DMF
3	4	4200	r.m=1.25 6 hrs, 50°C	240 min 65°C	60,1	3.0	58	254	As in example 1 with depolymerization to 4200 Da
4	5	8000	r.m=1.25 6 hrs, 50°C	240 min 65°C	150	2.0	98	n.d	As in example 1 with depolymerization to 8000 Da
5	6	20000	r.m=1.25 6 hrs, 50°C	240 min 65°C	135	1.0	n.d	725	As in example 1 without depolymerization
6	7	15000	r.m=1.25 6 hrs, 50°C	240 min 65°C	180	1.2	n.a	n.a	As in example 1 with depolymerization to 15000 Da
7	8	20000	Supersolf 18 hrs, 50°C	150 min 65°C	45	0.6	n.d	n.d	Supersulphation and 6O-sulphation as in WO 01/72848 without depolymerization
8	9	6000	Supersolf 18 hrs, 50°C	150 min 65°C	31,5	0.8	n.d	n.d	Supersulphation and 6O-sulphation as in WO 01/72848 with final depolymerization
9	10	6500	r.m=5 3 hrs, 50°C	60 min 65°C	89	2.5	73.9	395	As in example 1, with differences in step e)

10	11	6500	r.m=0.6 8 hrs, 50°C	30 min 65°C	101	2.5	n.d	423	As in example 1, with differences in step e)
11	12	8500	r.m=1.25 6 hrs, 50°C	240 min 65°C	300	1.8	94.2	n.d	As in example 1, with affinity column
12	13	6000	r.m=1.25 6 hrs, 50°C	240 min 65°C	65	1.0	n.d	n.d	As in example 1, with final depolymerization
13	14	6000	r.m=5 8 hrs, 50°C	240 min 65°C	75	1,2	n.d	n.d	As in example 1, with differences in step e)
14	15	6000	r.m=1.25 6 hrs, 50°C	240 min 65°C	5	n.d	n.d	n.d	As in example 1, without N-risulphation
15	16	6000	r.m=5 8 hrs, 50°C	240 min 65°C	8	n.d	n.d	n.d	As in example 13 without desulphation/ /6Osolfatazione/N-risolfatazione
16	17	6000	r.m=1.25 6 hrs, 50°C	240 min 65°C	95	2.5	n.d	n.d	As in example 1 with inversion of steps d) and e)
18	18	6000	r.m=1.25 6 hrs, 50°C	240 min 65°C	92	2.5	n.d	n.d	As in example 1 with inversion of steps d) - g)

n.d.= not determined

r.m.= molar ratio between the sulphating agent and substrate

TABLE 3: Comparative test of Heparinase hydrolysis of biotechnological and extractive heparins

Sample	Time: 0	Time: 10'	Time: 1 hour	Time: 2 hours	Time: 20 hours
Extractive heparin HMW (13600 Da)	12788 Da	8930 Da	4191 Da	5007 Da	4082 Da
Extractive heparin LMW (Fraxiparina)	5718 Da	4602 Da	nd	4187 Da	3417 Da
Biotechnological heparin MW=8500 Da (Ex. n°10)	8546 Da	7636 Da	7932 Da	8453 Da	7868 Da
Biotechnological heparin MW=10000 Da (Ex. n° 10 + depolym. to 10000 Da)	10087 Da	9979 Da	10978 Da	9993 Da	10479 Da

Table 4: Release assay of TFPI factor from HUVEC cells in vitro

Sample	TFPI released in medium (ng/ml)
Control (only culture medium)	0,5-0,8
Unfractionated extractive heparin (Vister, Pfizer) (1IUaXa/ml)	2,4-2,6
Biotechnological heparin 6000 Da (ex. n°1) (1IUaXa/ml)	2,5-3,7
Biotechnological heparin 8500 Da (ex. n°10) (1IUaXa/ml)	3.0-4,7

5

Table 5: Inhibition assay of protease generation (thrombin and Xa factor).

Sample	% Inhibition of Xa factor production		% Inhibition of thrombin production	
	intrinsic	extrinsic	intrinsic	extrinsic
Extractive LMWH heparin	50	90	0	0
Biotechnological heparin 6000 Da (ex. n°1)	80	90	65	80

Table 6: affinity assay for PF4 factor

Sample	% residual Xa activity in PF4 presence
Unfractionated extractive heparin (UFH)	45
Low molecular weight extractive heparin (LMWH)	65
Biotechnological heparin 4200 Da (ex. n°3)	90
Biotechnological heparin 8500 Da (ex. n°10)	70

CLAIMS

1. Process for the preparation of glycosaminoglicans-sulfates derived from acetyl N-heparosan comprising the following steps:

a) N-deacetylation and N-sulphation of a N-acetylheparosan polysaccharideisolated from natural or recombinant bacterial strain;

b) enzymatic epimerization by means of glucuronil C5-epimerase enzyme;

c) partial O-sulphation combined to partial O-desulphation;

d) partial 6-O sulphation;

e) N-resulphation

further comprising a controlled depolymerization step, alternatively performed after step b), c) or d), and wherein said process is characterized by the fact that the partial O-sulphation in step c) is performed for less than 10 hours and with a molar ratio between the sulfating agent and the N-acetyl heparosan lower than or equal to 5 and by the fact that the partial 6O-sulphation (step d) is performed for a time equal or lower than 2 hours and using a molar ratio between sulphating agent and the hydroxyl groups of the N-acetyl heparosan lower than or equal to 2.

2. The process according to claim 1 wherein steps d) and e) are performed in an inverted order.
3. Process according to claims 1-2, wherein intermediate depolymerization is carried out after step b) of epimerization.
4. Process according to claims 1-2 wherein the O-sulphation according to step c) is performed with a molar ratio between the sulfating agent and the N-acetyl heparosan lower than 2.5.
5. Process according to claim 4 wherein said molar ratio is equal or lower than 1.5.
6. Process according to claims 4-5 wherein the partial O-sulphation (step d) is performed for a time equal or lower than 6 hours.
7. Process according to claims 1-6 wherein the partial O-sulphation (step d) is carried out using a molar ratio between the sulphating agent and the hydroxyl groups of N-acetyl heparosan lower than or equal to 1.5.

8. Process according to claim 7 wherein the partial 6O-sulphation ((step d) of the process) is performed for a time equal to or lower than 60 minutes.
9. Process according to claim 8 wherein sulphation is carried out for a time equal or lower than 30 minutes.
10. Process according to claims 1-2 wherein the N-acetyl heparosan polysaccharide in step a) is isolated from E.coli K5.
11. Process according to claims 1-10 characterized by the fact that it comprises a further affinity selection step f) on matrixes binding antithrombin III or fragments thereof.
12. Process according to claims 1-11 wherein the partial sulphation according to step c) and the partial 6O-sulphation according to step d) are carried out using a sulphating agent selected from the group consisting of: triethylamine-SO3, trimethylamine-SO, pyridine-SO3 into an aprotic solvent.
13. Process according to claim 12, wherein said aprotic polar solvent is non-donor of formyl groups.
14. Process according to claim 12, wherein said aprotic polar solvent is chosen among: tetramethylensolfone, 2.4-Dimethylsulfolane or N,N-dimethylacetamide or N,N,-diethylacetamide.
15. Process according to claims 1-14 wherein the partial 6O-sulphation (step d) is performed at a temperature comprised from 4°C to 30°C.
16. Process according to claim 14 wherein said temperature is comprised from 10°C to 25°C.
17. Process according to claims 1-16 wherein the intermediate controlled depolymerization is performed by chemical or physical methods.
18. Process according to claim 17, wherein said physical methods comprise a gamma ray treatment and wherein said chemical methods comprise: a treatment with nitrous acid or salts thereof, or a beta-elimination or a periodic acid or a free radicals treatment.
19. Process according to claim 18, wherein the depolymerization is performed by treatment with nitrous acid or salts thereof.
20. Process according to claim 19 wherein the ratio between the nitrous acid or salts thereof and the polysaccharide is comprised from 1 to 100 mg salt per

gram of polysaccharide and the reaction is performed at a temperature comprised from 4 to 10°C.

21. Process according to with claim 20 wherein the controlled depolymerization is performed for a time lower than 30 minutes and in the presence of nitrous acids or salts thereof.

22. Process according to claim 21 wherein the nitrous acid salt is sodium nitrate.

23. Process according to claims 17-22, wherein depolymerization is ended by addition of a molar excess of borohydride.

24. Process for the preparation of sulphated glycosaminoglycans derived from acetyl N-heparosan comprising the following steps:

a) N-deacetylation and N-sulphation of a N-acetylheparosan polysaccharide isolated from a natural or a recombinant bacterial strain,

b) enzymatic epimerization by glucuronyl C5 epimerase,

c) partial O-sulphation combined to partial O-desulphation,

d) partial 6-O sulphation,

further comprising an intermediate step of controlled depolymerization performed alternatively after step b), c) or d) wherein said process is characterized by the fact that partial O-sulphation in step c) is carried out for a time equal or lower than 10 hours and using a molar ratio between sulphating agent and the N-acetyl heparosan lower than or equal to 5 and in that the partial 6O-sulphation (step d) is performed for a time equal or lower than 2 hours and with a molar ratio between the sulphating agent and the N-acetyl heparosan lower than or equal to 2 and in that the O-sulphations in steps c) and d) are performed with a sulphating agent selected among: triethylamine-SO3, trimethylamine-SO, pyridine-SO_3 in an aprotic solvent.

25. Process according to claims 1-24 characterized in that the reaction of C-5 epimerization (step c) is carried out at a temperature below 35°C and wherein the glucuronyl C5-epimerase either extractive or recombinant is immobilized on a stationary phase.

26. Process according to claim 25 wherein said recombinant C5 epimerase is a mouse enzyme expressed in insect or in yeast cells.

27. Process according to claim 25 wherein said temperature is comprised from 15°C to 30°C.

28. Process according to claim 27 wherein said temperature is comprised from 20°C to 25°C.

29. Process according to claim 25 wherein the stationary phase is a polystyrenic or polymethacrylic resin with hepoxidic or diolic groups activated with CNBr and that the C5-epimerase immobilization is carried out in a buffer comprising NaHCO3 in concentration from 100 to 300 mM or in phosphate buffer in concentration from 10 to 50 mM at pH from 7,0 to 8,3 at a temperature comprised from 4 to 25°C for a time comprised from 12 to72 hours.

30. Process according to claims 25-29 wherein the epimerization in step c) is carried out in HEPES buffer comprising: EDTA from 10 to 30 mM, $CaCl_2$ from 70 to 150 mM and with a pH from 5,5 to 8,0.

31. Modified N-acetyl heparosan obtainable by the process according to claims 1-30.

32. Modified N-acetyl heparosan according to claim 31 with a molecular weight lower than or equal to 15000 Da.

33. Modified N-acetyl heparosan according to claim 32 with a molecular weight comprised from 3000 to 9000 Da.

34. Modified N-acetyl heparosan obtainable by the process according to claim 13 characterized by the absence of formyl groups in the molecule.

35. Modified N-acetyl heparosan according to claim 31-34 characterized by the fact it carries at the reducing extremity a residue of sulphated 2,5 anhydromannitol.

36. N-acetyl heparosan according to claim 35 wherein the hydroxyl groups in position 1, 3 and 6 of anhydromannitol are partially sulphated.

37. N-acetyl heparosan according to claim 36 wherein the anhydromannitol is partially sulphated on the hydroxyls in position 1 and 6.

38. K5OS6OSNS-epi N-acetyl heparosan according to claim 37 wherein the sulphatation degree of the hydroxyl groups in position 1 is comprised from 20% to 85%.

39. K5OS6OSNS-epi N-acetyl heparosan according to claim 37 wherein the sulphatation degree of the hydroxyl groups in position 6 of the glucosamine is higher than 40%.

40. K5OS6OSNS-epi N-acetyl heparosan according to claim 39 wherein said sulphatation degree is comprised from 50 to 85%.

41. N-acetyl heparosan according to claim 36 wherein the sulphatation degree of the hydroxyl groups in position 3 of the glucosamine is lower than 60%.

42. K5OS6OSNH$_2$,epi obtainable by the process according to claim 24.

43. K5OSNH$_2$-epi obtainable by the process according to claim 24 steps a)-c).

44. Modified N-acetyl heparosan according to claims 31-41 characterized by an anti-factor Xa activity measured in presence of plasma equal to or higher than 50 IU/mg.

45. Modified N-acetyl heparosan according to claim 44 characterized by a ratio between the factor Xa and the factor IIa inhibition activities equal or higher than 1.0.

46. Modified N-acetyl heparosan according to claim 44 characterized by a TFPI activation activity equal or higher compared to extractive heparins.

47. Modified N-acetyl heparosan according to claim 44 characterized in that it has a heparinase I resistance higher than extractive heparins.

48. Modified N-acetyl heparosan according to claim 44 characterized in that it inhibits the generation of thrombin and Xa factor proteases.

49. Modified N-acetyl heparosan according to claim 44 characterized in that has a lower affinity for PF4 factor as compared to extractive heparins.

50. Modified N-acetyl heparosan according to claim 44 characterized by a HCII activity higher than extractive heparins.

51. Modified N-acetyl heparosan according to claims 44-50 characterized by the presence of multiple signals in a ^{13}C NMR spectrum in excess as compared to the signals of anhydromannitol in the region comprised from 79 to 89 ppm, by the absence of signals at 51 and 165 ppm and by the absence of signals in region at ppm 7-9,5 of ^{1}H-NMR spectrum.

52. K5 OS 6OS N-epi according to claim 51 having a ^{1}H-NMR spectrum corresponding to figure 2.

53. K5 OS 6OS N-epi according to claim 51 having a ^{13}C-NMR spectrum corresponding to figure 7.

54. Modified N-acetyl heparosan according to claims 31-41 and 44-53 for pharmacological use.

55. Use of the product according to claim 54 for the preparation of a medicament with heparin-like antithrombotic and anticoagulant activity.

56. Use of the product according to claim 54 for the preparation of a medicament medicines with profibrinolytic and antiaggregant activity.

57. The use according to claim 55 for the preparation of a medicament for the prophylaxis and the treatment of unstable angina, of myocardial heart attack, of deep venous thrombosis, of lungs embolism and of ischemic events.

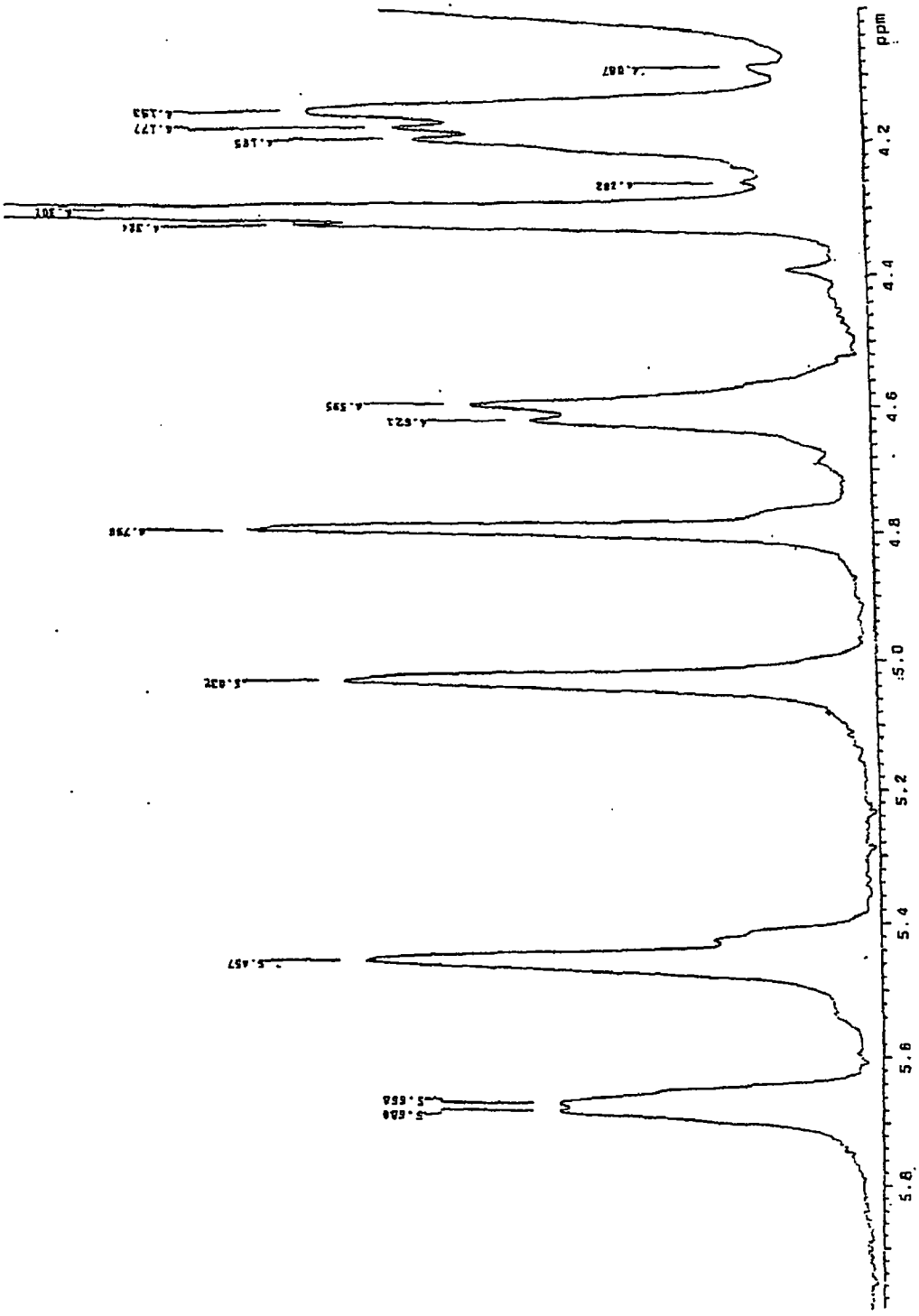
58. Use according to claims 55-56 for the preparation of a medicament for the treatment of sepsis and of its complications such as disseminated intravascular coagulation (CID).

59. Use according to claim 56 for the preparation of a medicament for the prophylaxis and the treatment of unstable angina, of myocardial acute heart attack, of venous and arterious thrombosis, of lungs embolism, of ischemic events, of arteriosclerosis.

60. Use according to claims 54-55 for the preparation of a medicament for the prophylaxis and the treatment of thromboembolytic events due to congenital or acquired lack of antithrombin III.

61. Pharmaceutical composition comprising as the active principle anyone of the products according to claims 31-41 and 44-53 in combination with suitable excipients and/or diluents.

62. Pharmaceutical composition according to claim 61 in composition suitable for oral adminstration.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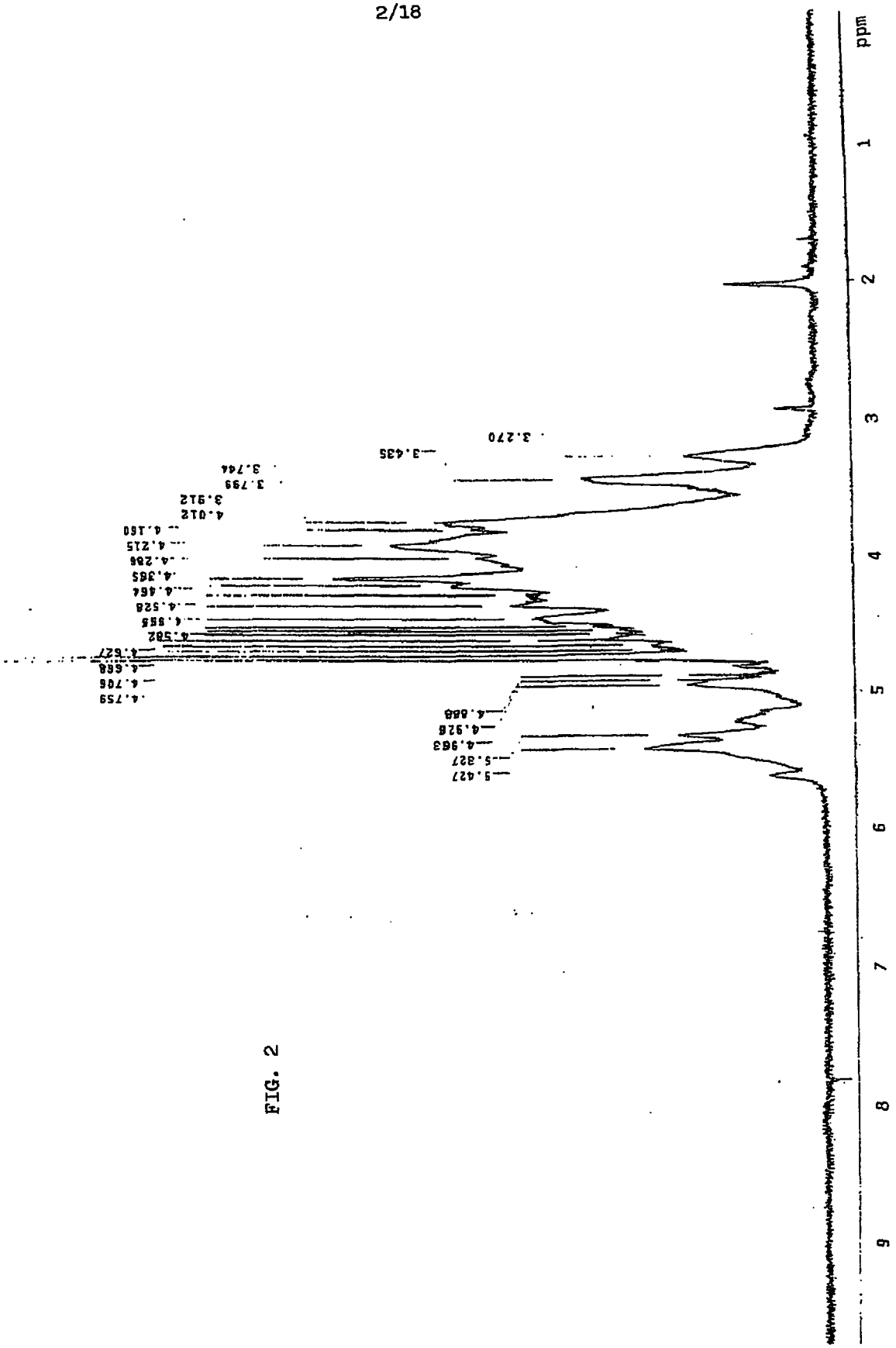
5.8

FIG 1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5.427

FIG. 2

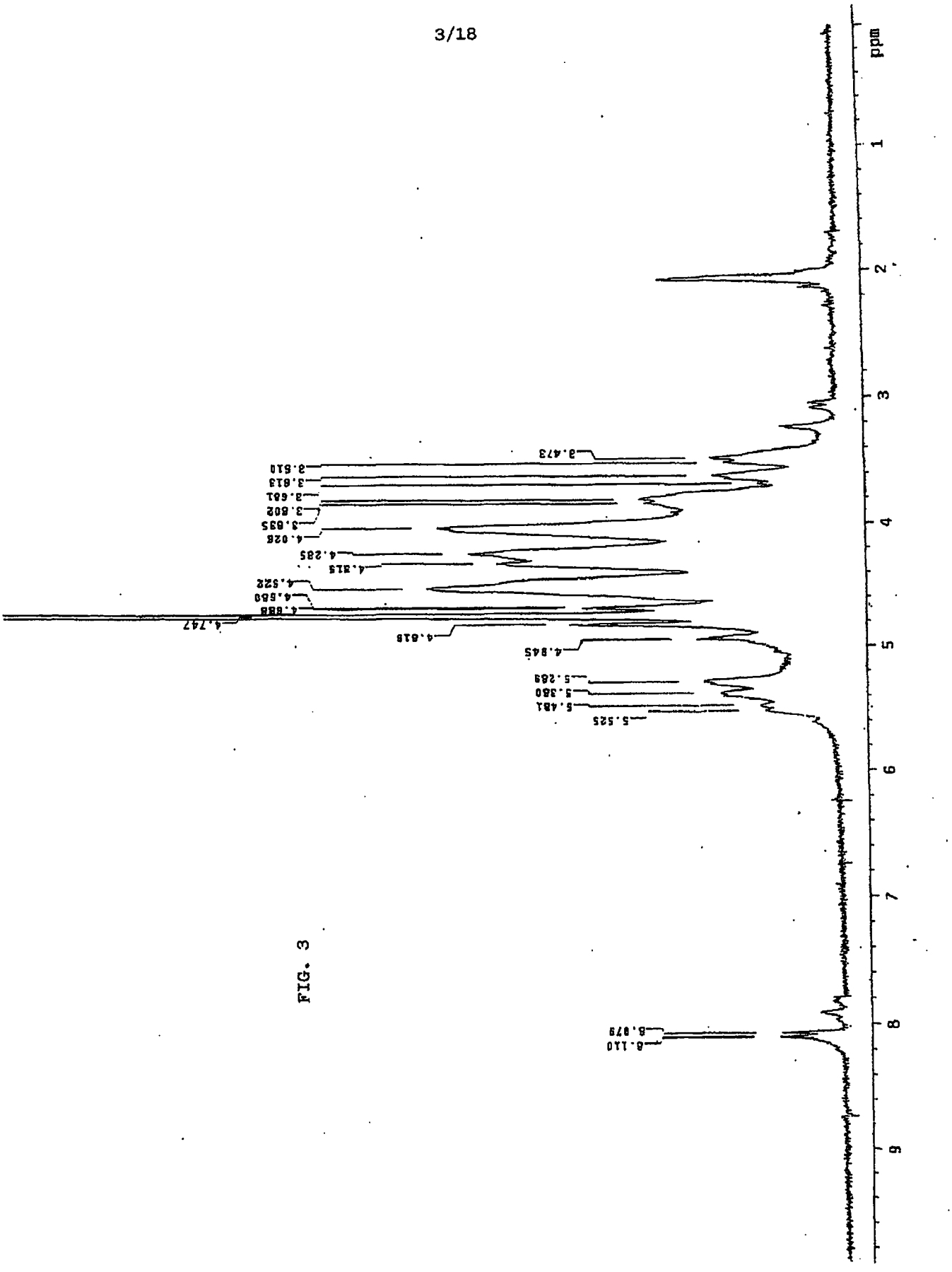
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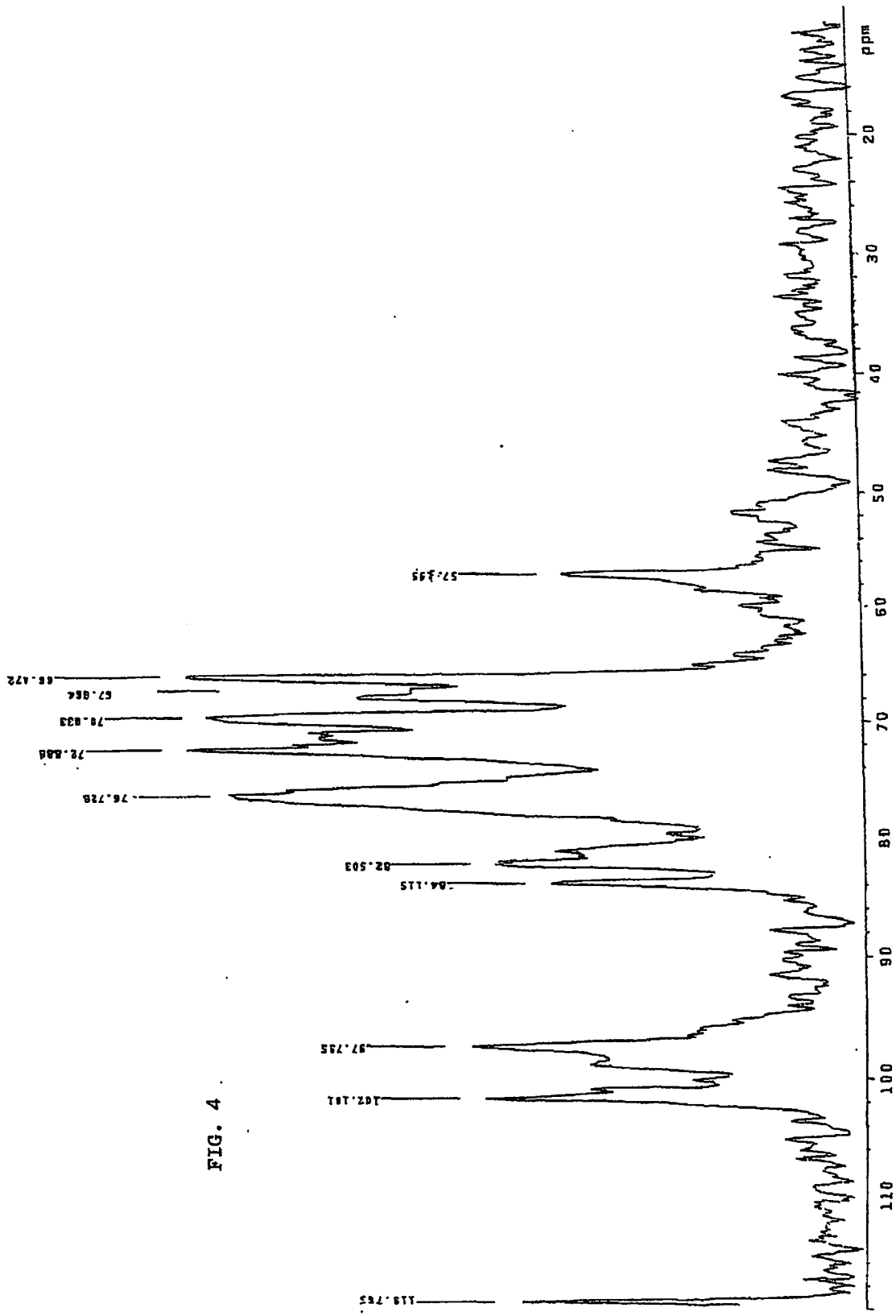
FIG. 3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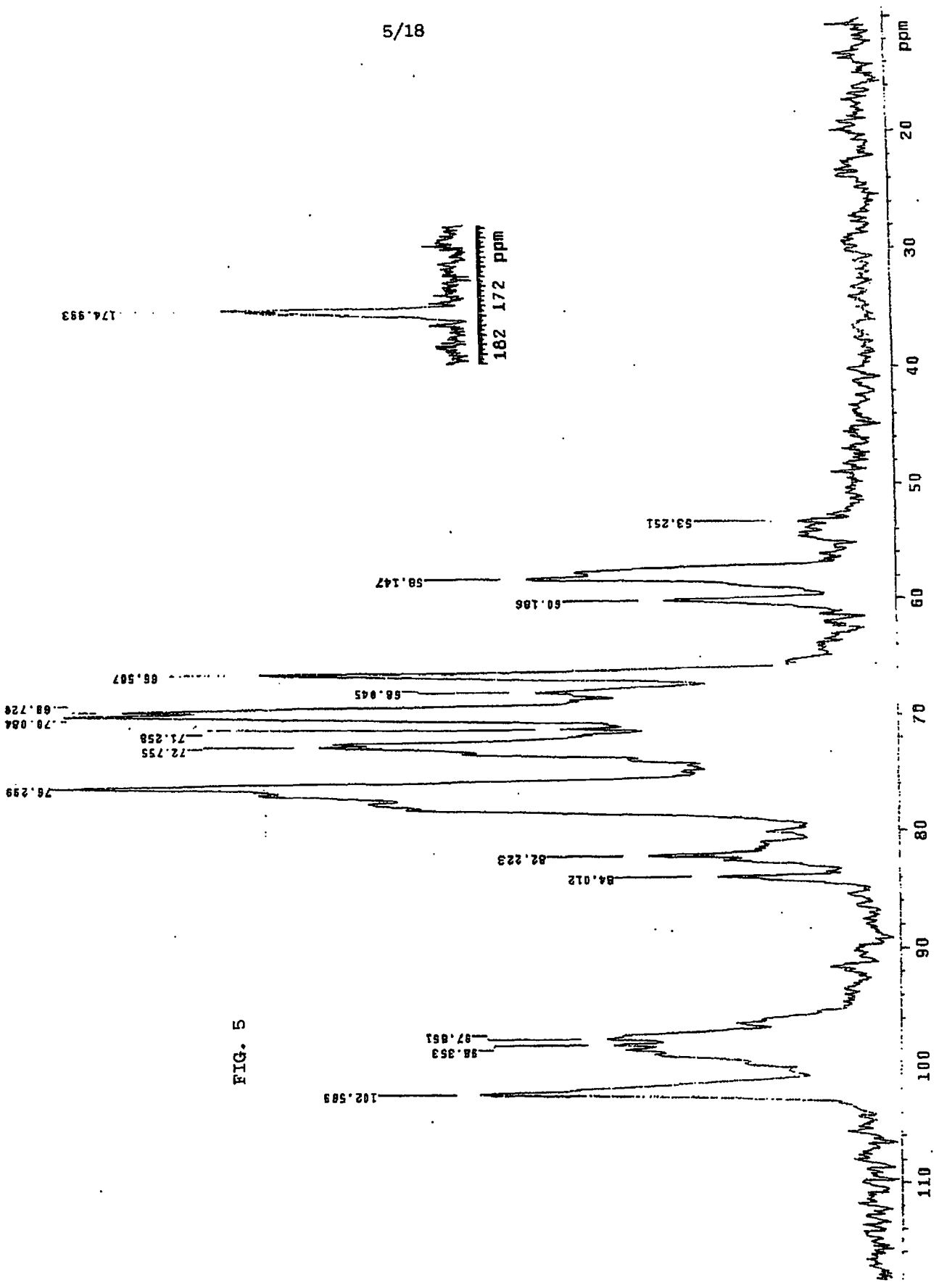
97.795
102.181
118.765

FIG. 4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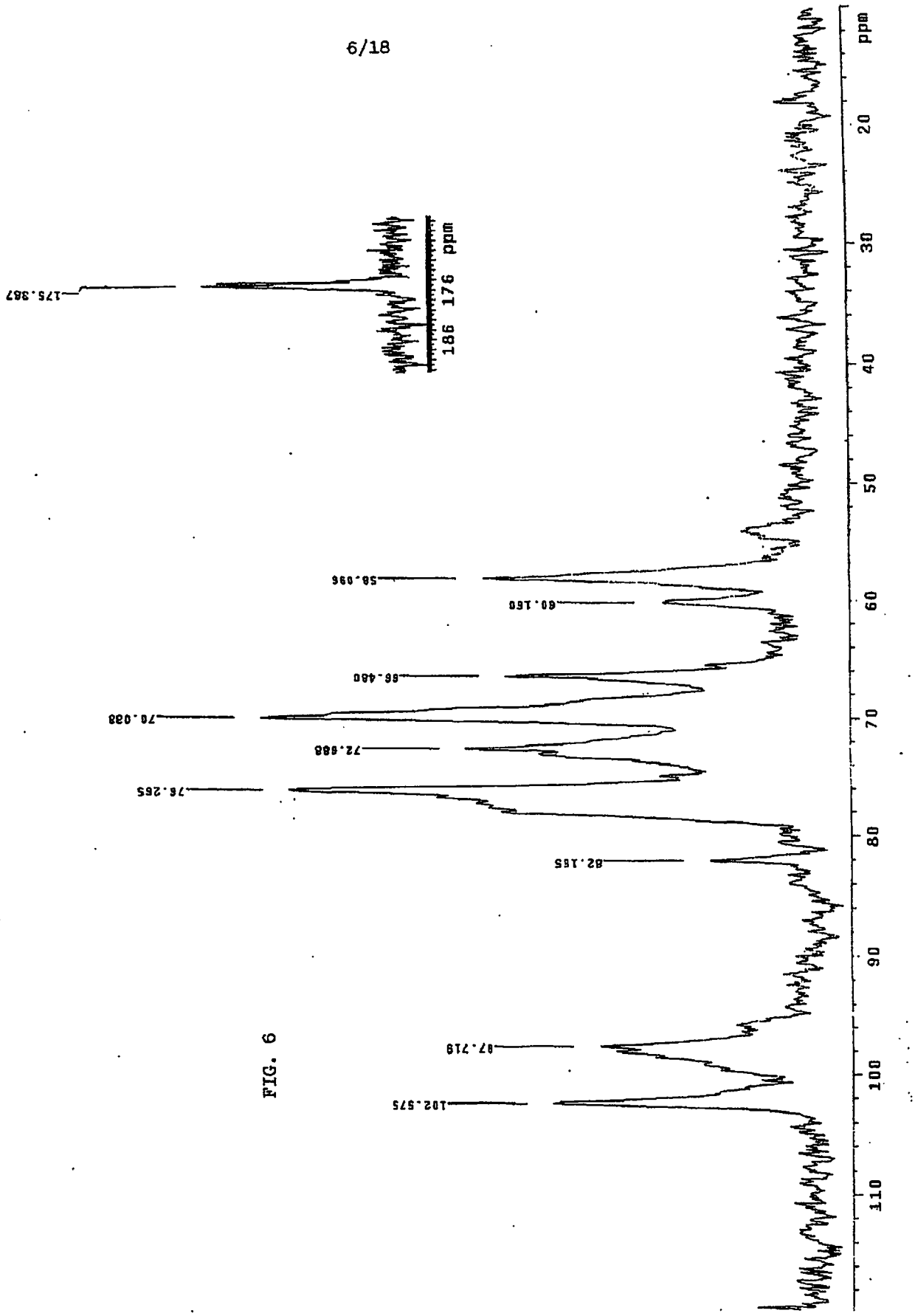
71.258
70.084
68.728
66.507
66.045
60.186
58.147
53.251

FIG. 5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70.028
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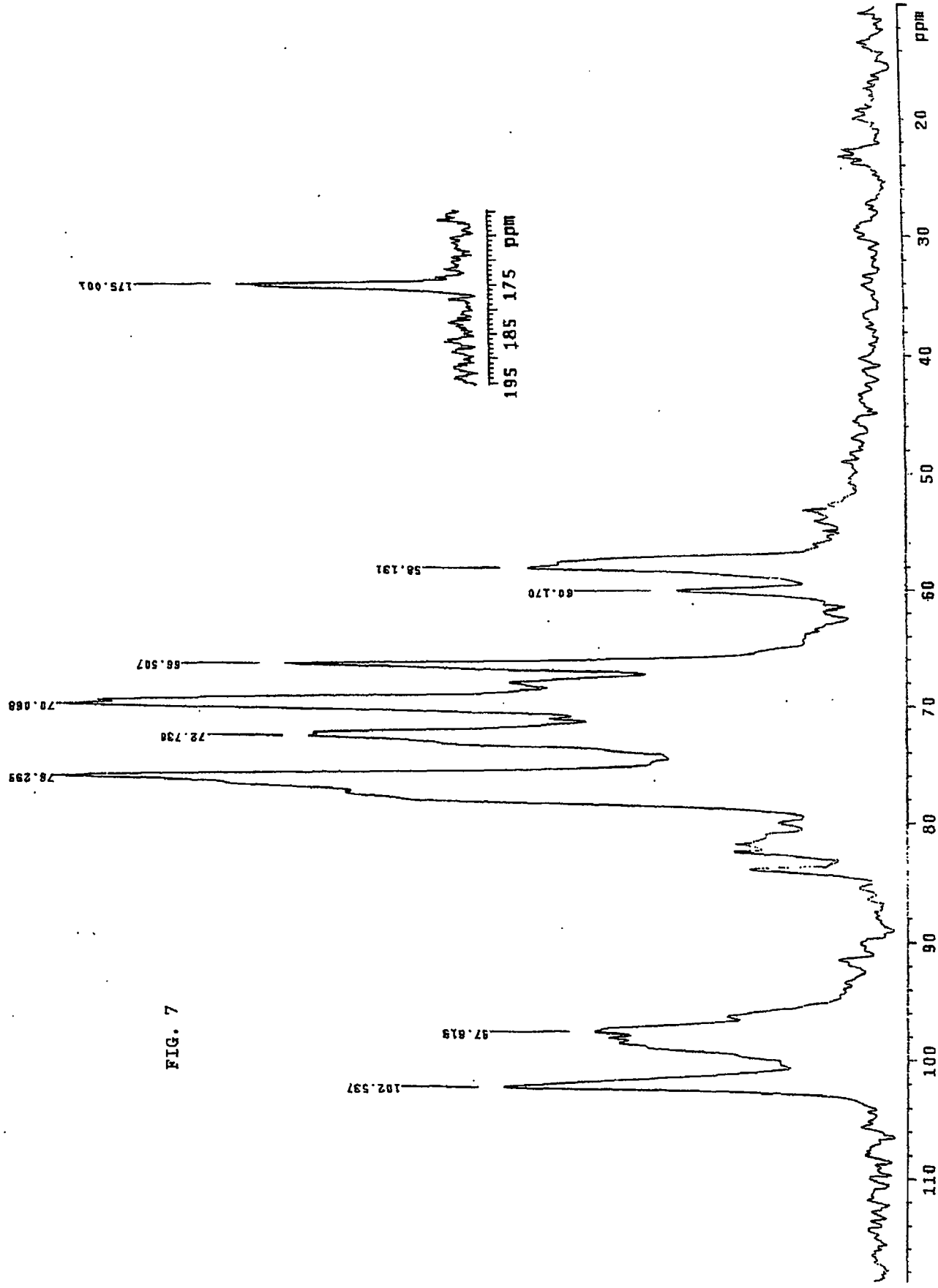
76.265
82.165
87.719
102.575

FIG. 6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97.619
102.537

FIG. 7

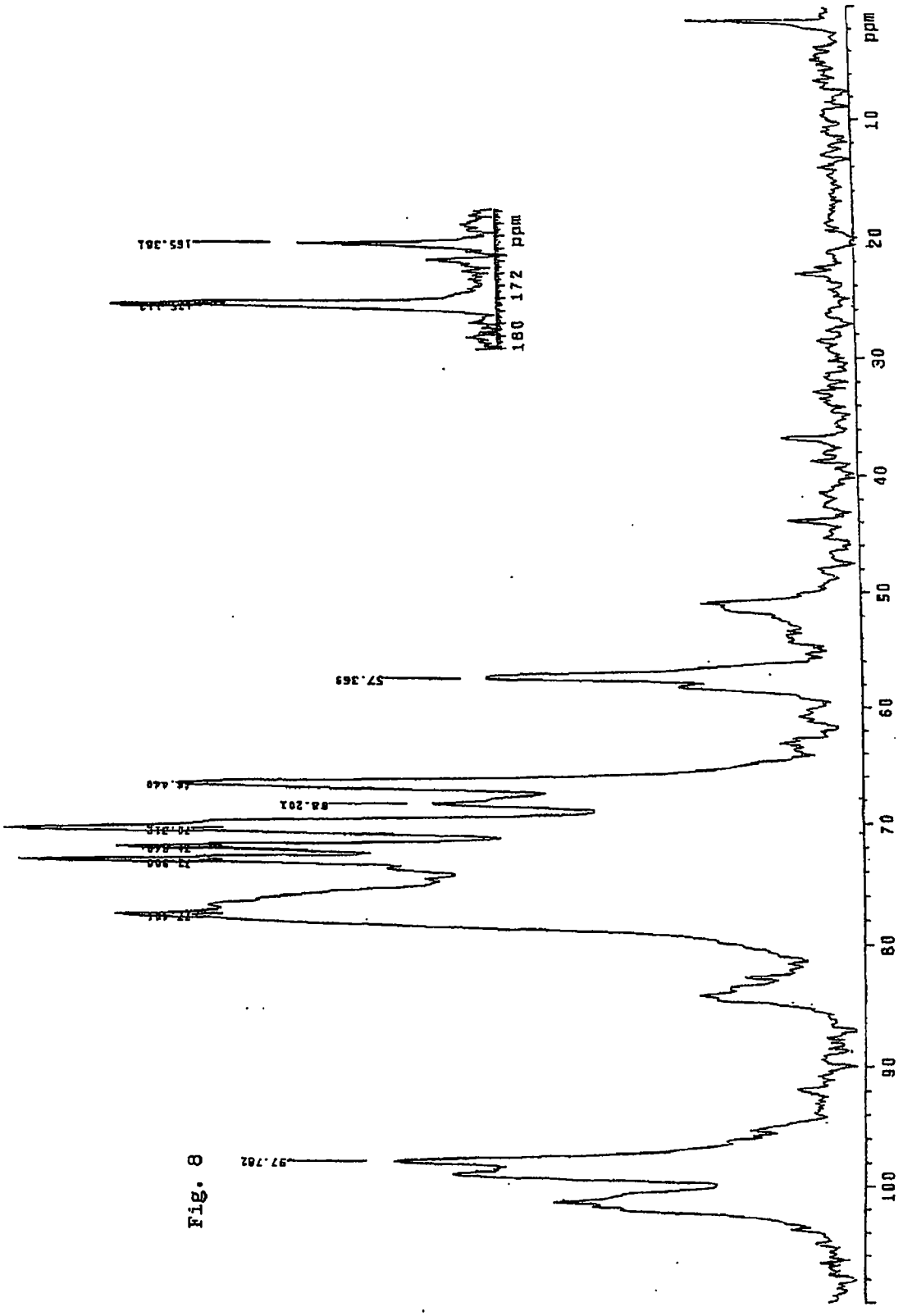
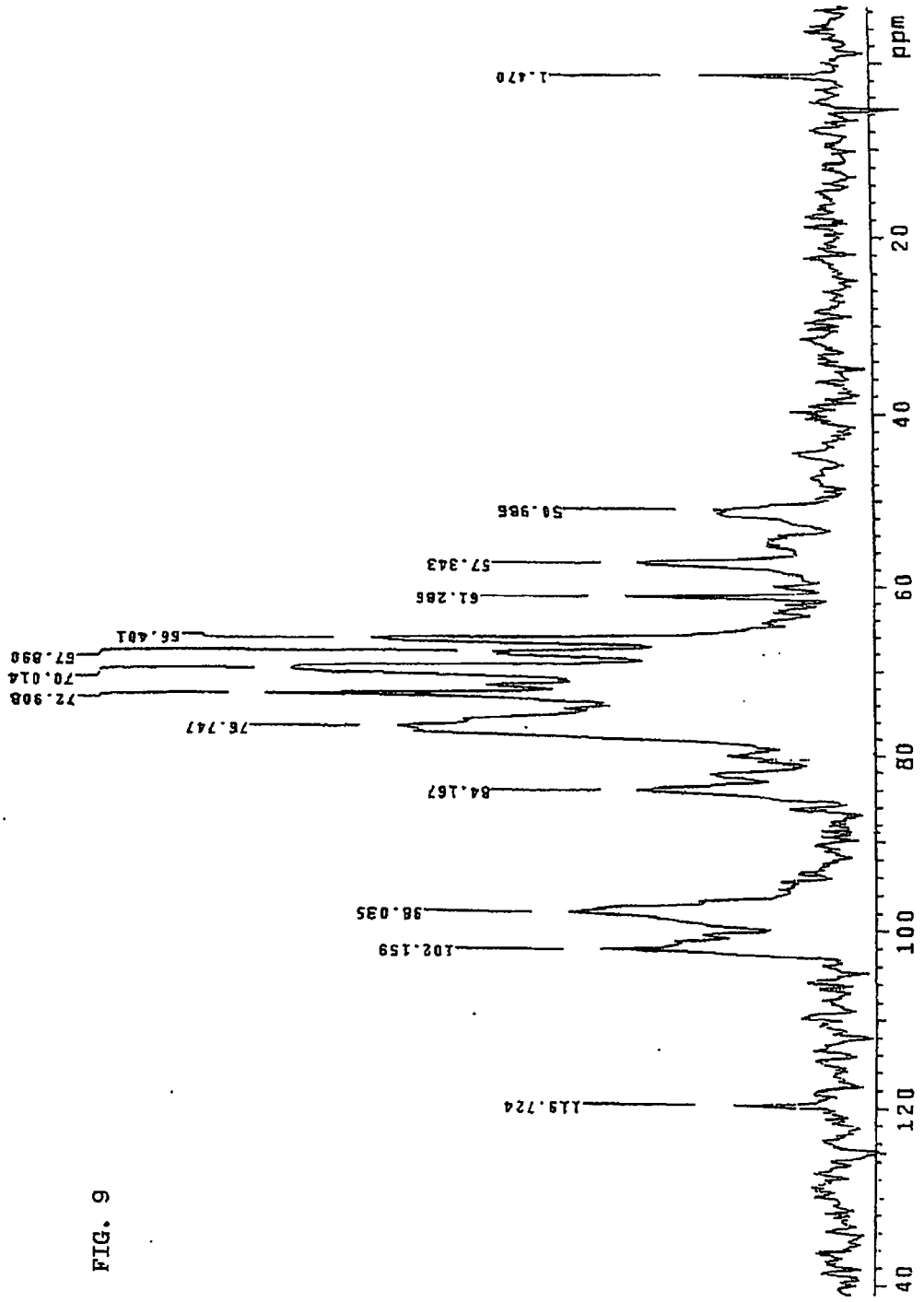


Fig. 8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40
60
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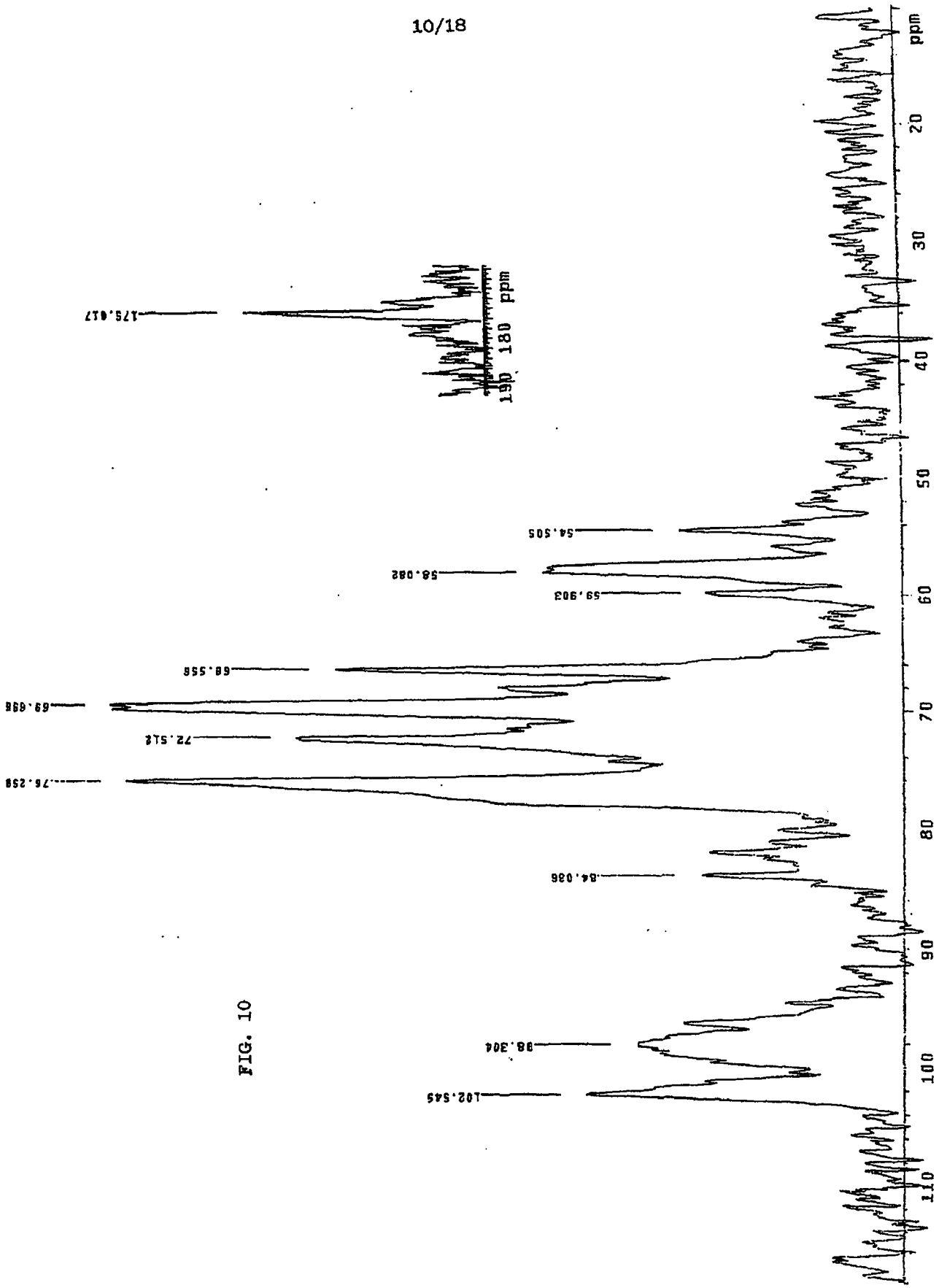
100
120
140

FIG. 9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ppm
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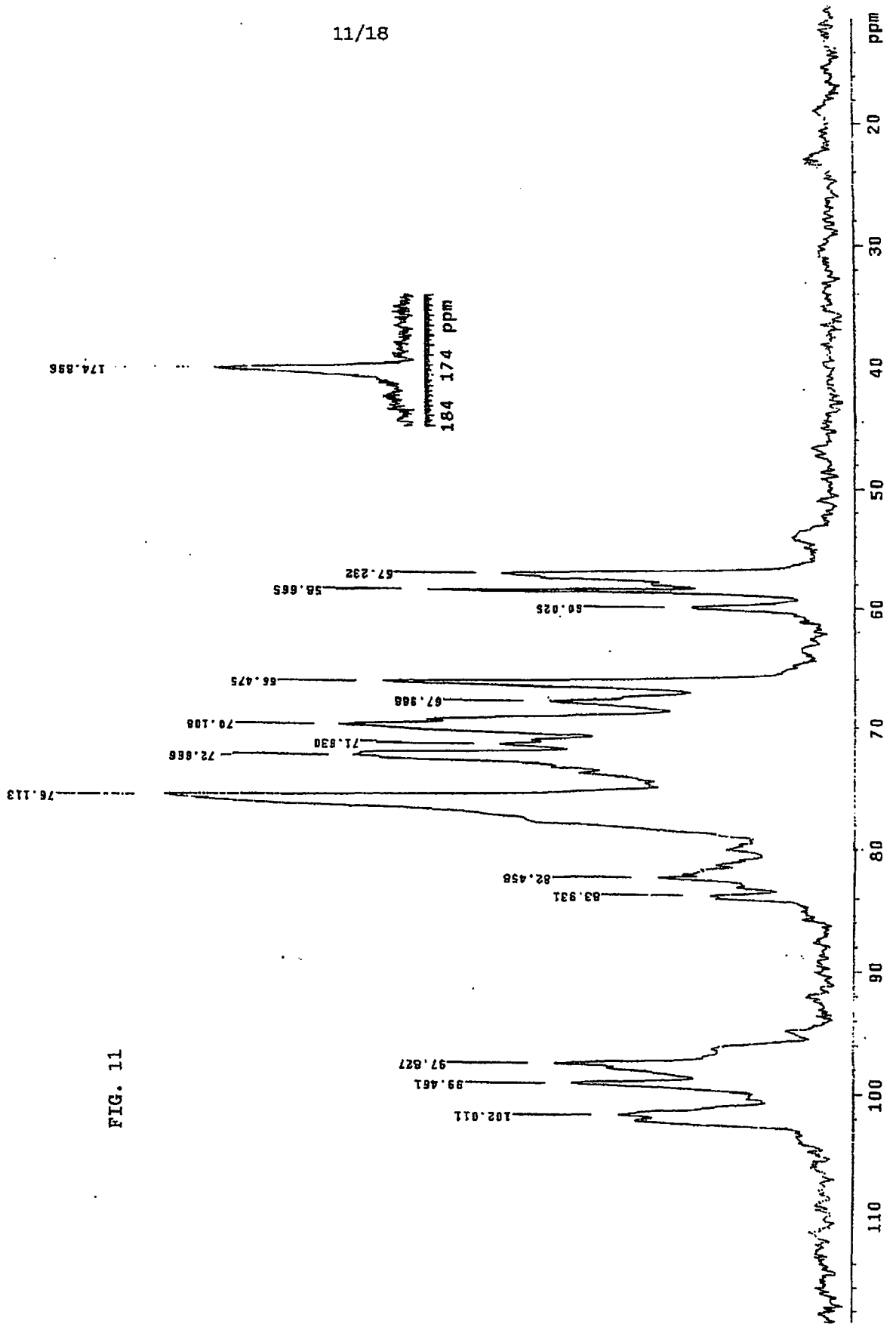
100
110

FIG. 10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184 174 ppm
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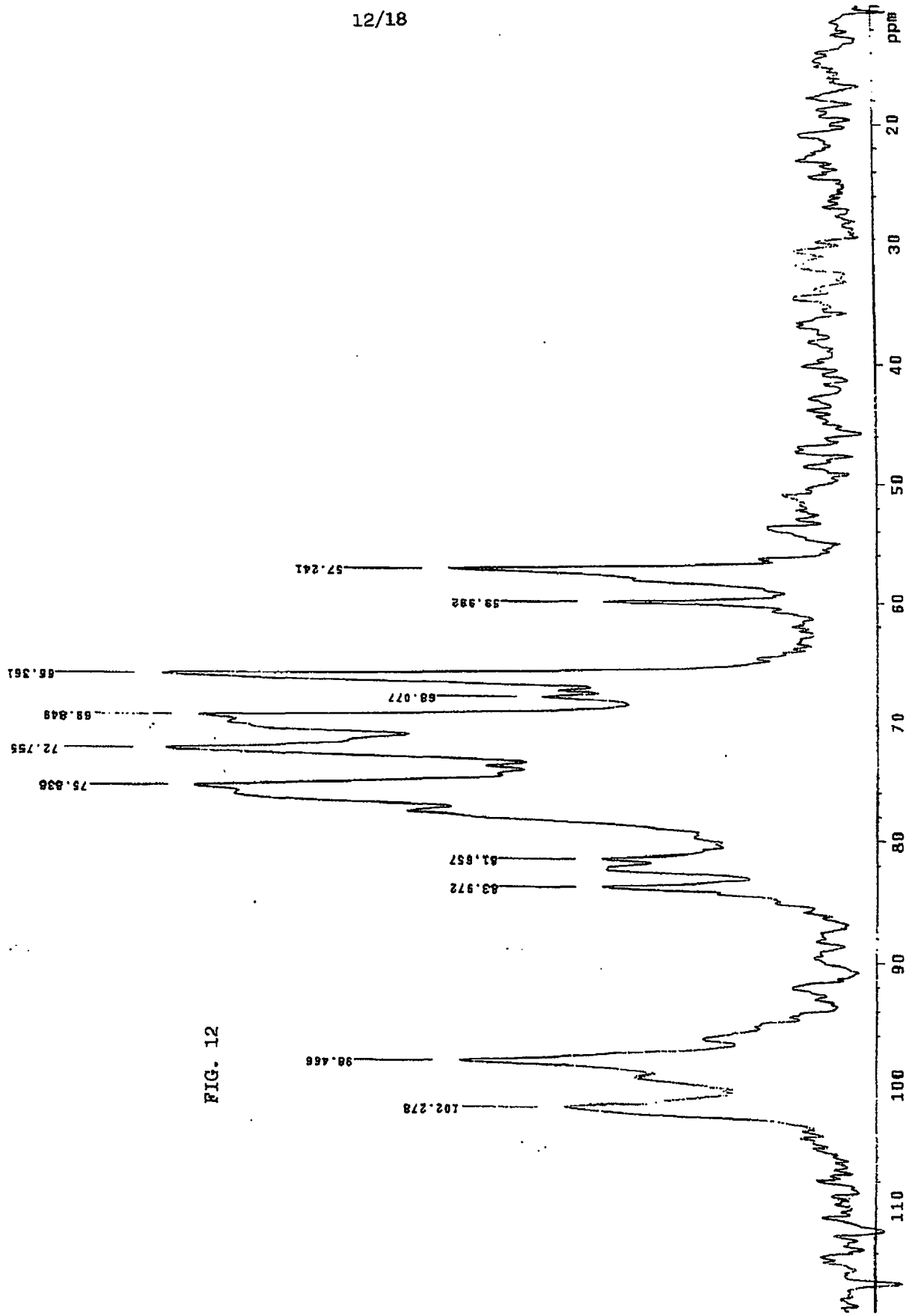
97.827
99.461
102.011

FIG. 11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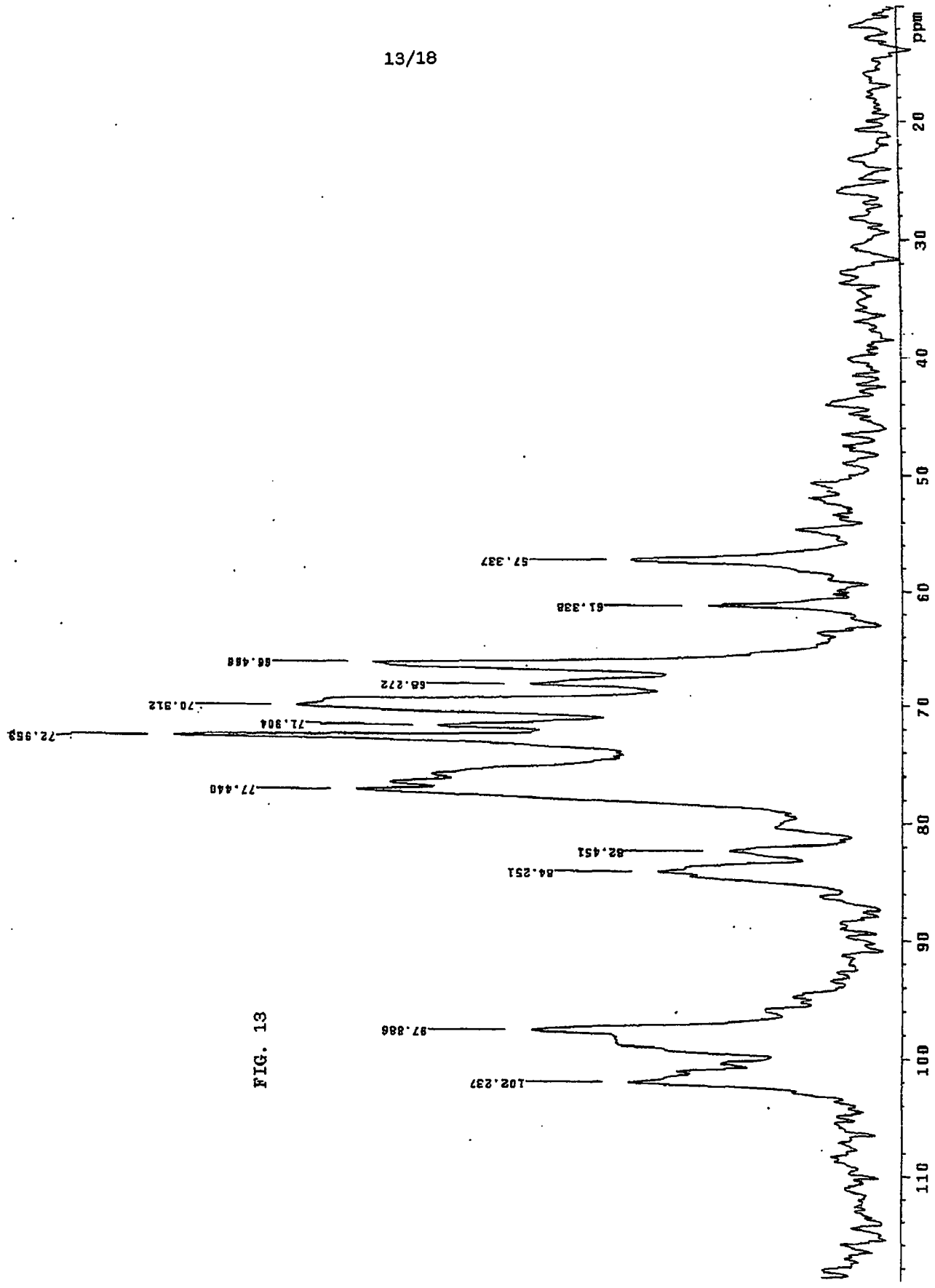
98.466
102.278

FIG. 12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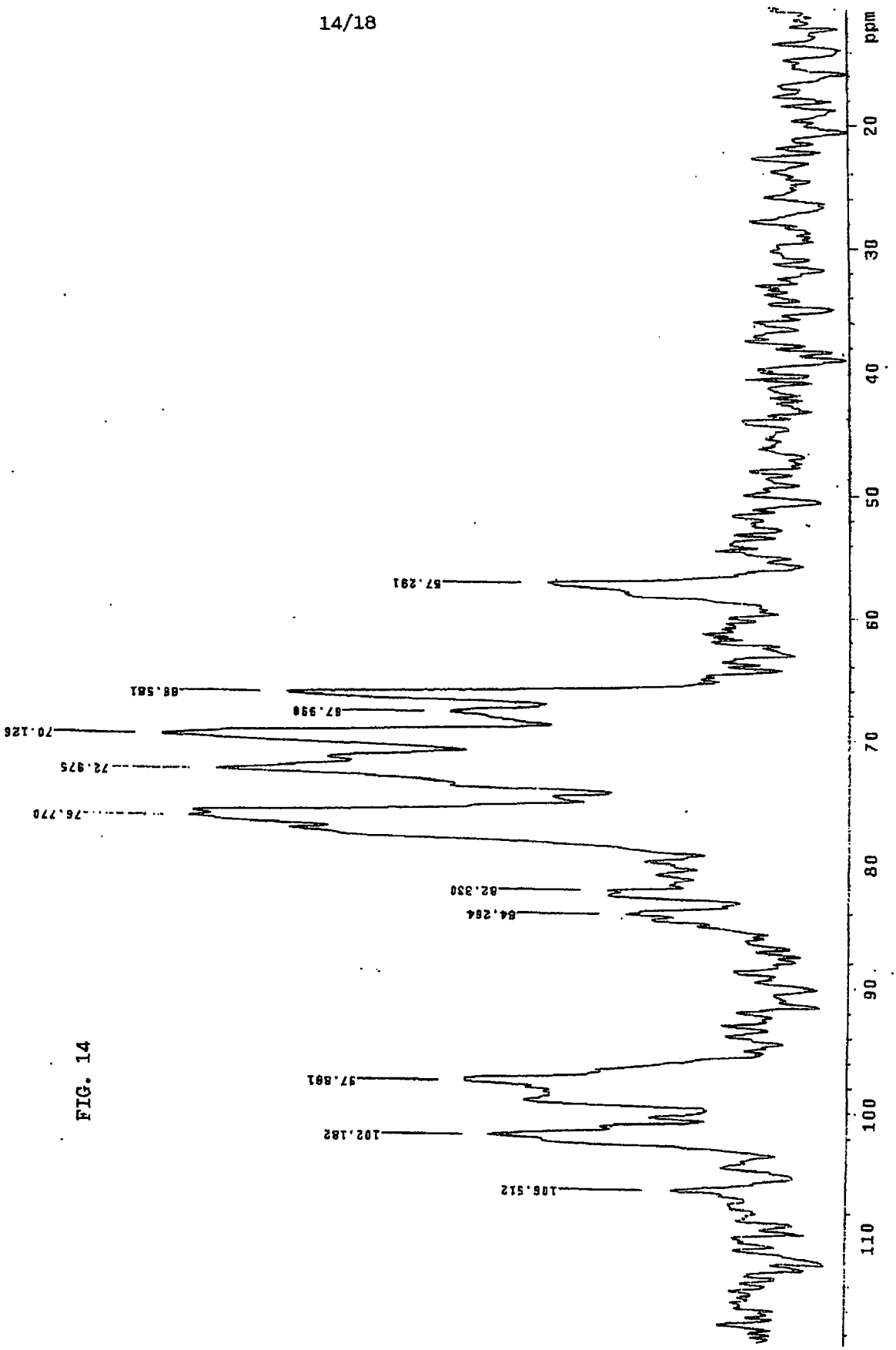
102.237

FIG. 13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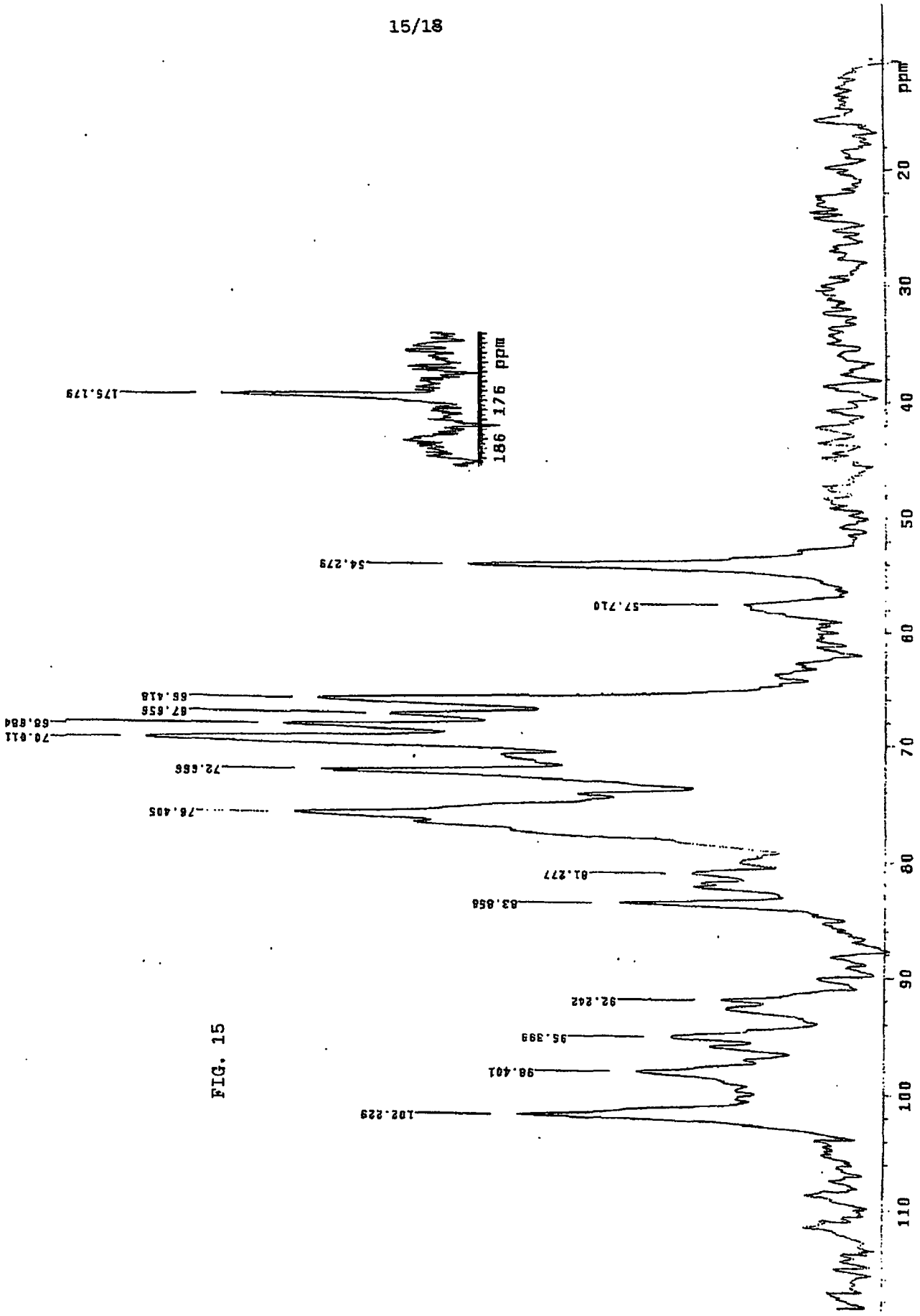
106.512

FIG. 14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102.229

FIG. 15

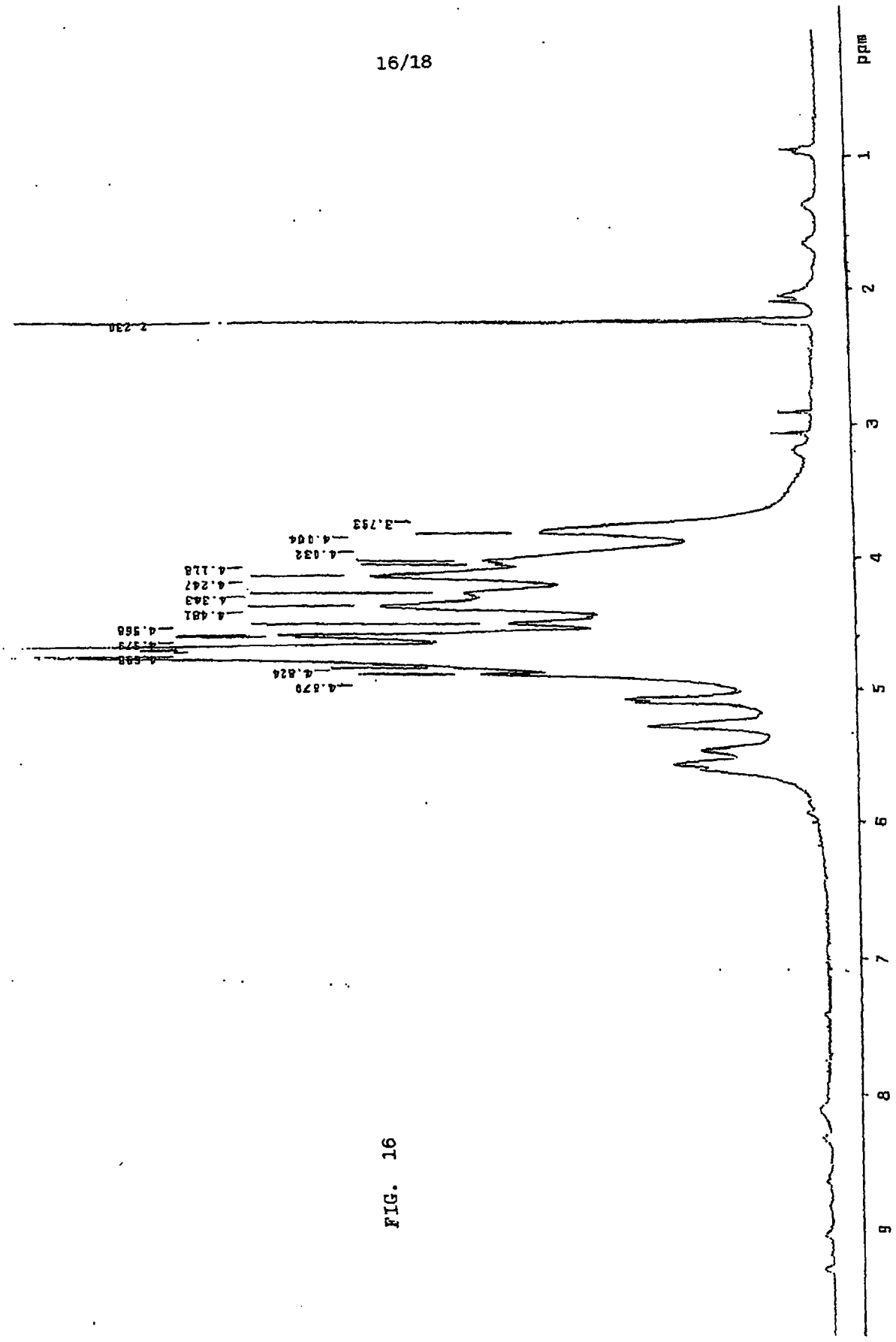
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FIG. 16

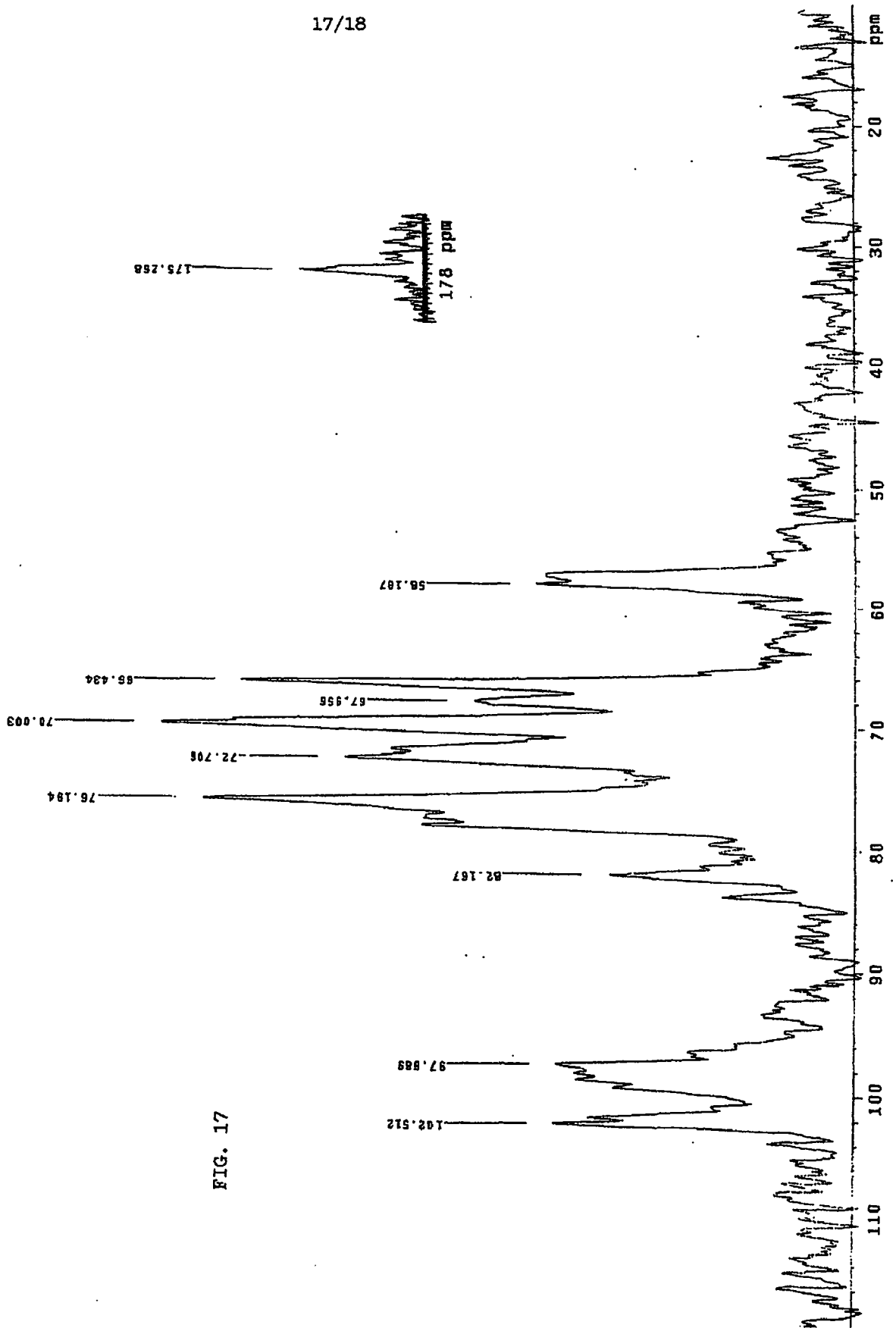
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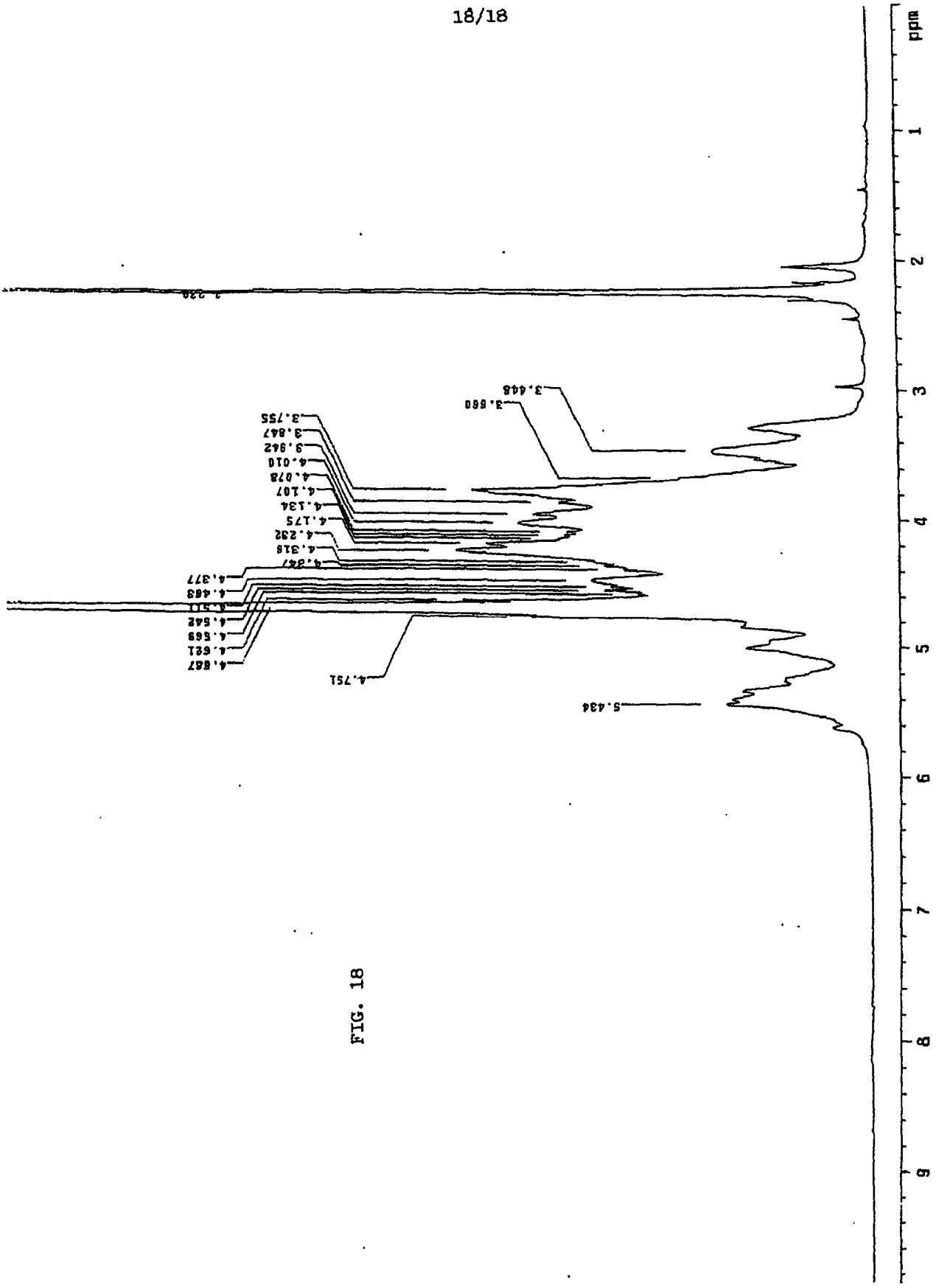
FIG. 17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4.292
4.318
4.387
4.377
4.488
4.512
4.542
4.569
4.621
4.887
4.751
5.434

FIG. 18