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(54) Title: **HYALURONIC ACID LINKED WITH A POLYMER OF AN ALPHA HYDROXY ACID**

(57) Abstract: The invention concerns a product comprising hyaluronic acid or a salt thereof, wherein the hyaluronic acid has been partially or fully linked or crosslinked with a polymer of an alpha hydroxy acid. The invention also concerns manufacture of the product, uses of the product of the invention in the field of biodegradable plastic materials for the preparation of sanitary and surgical articles, in the pharmaceutical and cosmetic fields; including the various articles made with the same in such fields.



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TITLE: Hyaluronic acid linked with a polymer of an alpha hydroxy acid**FIELD OF INVENTION**

The invention concerns a product comprising hyaluronic acid or a salt thereof, wherein the hyaluronic acid has been partially or fully linked or crosslinked with a polymer of an alpha hydroxy acid. The invention also concerns manufacture of the product, uses of the product of the invention in the field of biodegradable plastic materials for the preparation of sanitary and surgical articles, in the pharmaceutical and cosmetic fields; including the various articles made with the same in such fields.

BACKGROUND

The most abundant heteropolysaccharides of the body are the glycosaminoglycans. Glycosaminoglycans are unbranched carbohydrate polymers, consisting of repeating disaccharide units (only keratan sulphate is branched in the core region of the carbohydrate). The disaccharide units generally comprise, as a first saccharide unit, one of two modified sugars - N-acetylgalactosamine (GalNAc) or N-acetylglucosamine (GlcNAc). The second unit is usually an uronic acid, such as glucuronic acid (GlcUA) or iduronate.

Glycosaminoglycans are negatively charged molecules, and have an extended conformation that imparts high viscosity when in solution. Glycosaminoglycans are located primarily on the surface of cells or in the extracellular matrix. Glycosaminoglycans also have low compressibility in solution and, as a result, are ideal as a physiological lubricating fluid, e.g., joints. The rigidity of glycosaminoglycans provides structural integrity to cells and provides passageways between cells, allowing for cell migration. The glycosaminoglycans of highest physiological importance are hyaluronan, chondroitin sulfate, heparin, heparan sulfate, dermatan sulfate, and keratan sulfate. Most glycosaminoglycans bind covalently to a proteoglycan core protein through specific oligosaccharide structures. Hyaluronan forms large aggregates with certain proteoglycans, but is an exception as free carbohydrate chains form non-covalent complexes with proteoglycans.

Numerous roles of hyaluronan in the body have been identified (see, Laurent T. C. and Fraser J. R. E., 1992, FASEB J. 6: 2397-2404; and Toole B.P., 1991, "Proteoglycans and hyaluronan in morphogenesis and differentiation." In: Cell Biology of the Extracellular Matrix, pp. 305-341, Hay E. D., ed., Plenum, New York). Hyaluronan is present in hyaline cartilage, synovial joint fluid, and skin tissue, both dermis and epidermis. Hyaluronan is also suspected of having a role in numerous physiological functions, such as adhesion, development, cell motility, cancer, angiogenesis, and wound healing. Due to the unique physical and biological properties of hyaluronan, it is employed in eye and joint surgery and is being evaluated in other medical procedures.

The term "hyaluronic acid" is used in literature to mean acidic polysaccharides with different molecular weights constituted by residues of D-glucuronic and N-acetyl-D-glucosamine acids, which occur naturally in cell surfaces, in the basic extracellular substances of the connective tissue of vertebrates, in the synovial fluid of the joints, in the endobulbar fluid of the eye, in human umbilical cord tissue and in cocks' combs.

The term "hyaluronic acid" is in fact usually used as meaning a whole series of polysaccharides with alternating residues of D-glucuronic and N-acetyl-D-glucosamine acids with varying molecular weights or even the degraded fractions of the same, and it would therefore seem more correct to use the plural term of "hyaluronic acids". The singular term will, however, be used all the same in this description; in addition, the abbreviation "HA" will frequently be used in place of this collective term.

HA plays an important role in the biological organism, as a mechanical support for the cells of many tissues, such as the skin, tendons, muscles and cartilage, it is a main component of the intercellular matrix. HA also plays other important parts in the biological processes, such as the moistening of tissues, and lubrication.

HA may be extracted from the above mentioned natural tissues, although today it is preferred to prepare it by microbiological methods to minimize the potential risk of transferring infectious agents, and to increase product uniformity, quality and availability (WO 03/0175902, Novozymes).

HA and its various molecular size fractions and the respective salts thereof have been used as medicaments, especially in treatment of arthropathies, as an auxiliary and/or substitute agent for natural organs and tissues, especially in ophtalmology and cosmetic surgery, and as agents in cosmetic preparations. Products of hyaluronan have also been developed for use in orthopaedics, rheumatology, and dermatology.

HA may also be used as an additive for various polymeric materials used for sanitary and surgical articles, such as polyurethanes, polyesters etc. with the effect of rendering these materials biocompatible.

The preparation of a crosslinked HA or salt thereof, which is prepared by crosslinking HA with a polyfunctional epoxy compound is disclosed in EP 0 161 887 B1. Total or partial crosslinked esters of HA with an aliphatic alcohol, and salts of such partial esters with inorganic or organic bases, are disclosed in US 4,957,744.

US 6673919 B2 (Chisso Corp. pub. Date 06/01/2004) relates to a process for chemically modifying hyaluronic acid or a salt thereof by O-acetylation, alkoxylation, or crosslinking a complex consisting of hyaluronic acid or a salt thereof and a solution of a cationic compound.

FR 2707653 (Vetoquinol) relates to a conjugate between a biocompatible and biodegradable polymer and a molecule, especially a biologically active molecule containing

mobile hydrogen; a process for its preparation; and a pharmaceutical composition including this conjugate.

The present invention relates to a chemical grafting technology on hyaluronic acid (HA) using synthetic polymers and oligomers made of repeating units of alpha hydroxy acids, such as poly(lactic acid), also named polylactide, and any lactic acid-based polymers, stereocopolymers and copolymers, especially those with glycolic acid, but also with other copolymers such as copolymers with hydroxy caproic acid via ϵ -caprolactone, gluconic acid and chemically modified gluconic acid, malic acid, copolymers with low molecular weight segments that can lead to degradation by-products that are hydrosoluble and that can be eliminated via kidney filtration, such as low molecular weight poly(ethylene glycol)s, provided that they bear one or two carboxyl groups at chain ends, and that they provide hydrophobicity in the case of monoacids. Importantly, the methodology can be exploited either to derivatize HA by grafting or cross-linking, and the products could be used for technical, biomedical and pharmaceutical applications. The grafted HA is biodegradable, biocompatible and bioresorbable.

SUMMARY OF THE INVENTION

Derivatization of HA with poly alpha hydroxy acids, e.g. oligomers of lactic acid or glycolic acid, is employed to prepare a grafted HA structure that is more hydrophobic than HA itself. The resulting amphiphilic properties are desirable in cosmetic applications such as emulsion stabilization, skin moisturization and tightening, and film forming. Hydrogels or nanosized colloidal dispersions from such grafted materials could also be used for tissue augmentation, adhesion prevention, osteoarthritis and ophthalmology.

Only biocompatible metabolites will be released upon biodegradation of the grafted materials. The degradation by-products, such as lactic acid or glycolic acid, are metabolized by the body and completely eliminated, thus making the grafted product completely bioresorbable in the body.

Lactic acid is widely used in cosmetic formulations and poly(lactic acid) (PLA) is widely used in biomedical applications for tissue engineering, and also in pharmaceutical applications for drug delivery, e.g., using PLA microspheres and nanoparticles.

Poly(lactic acid) (acid chloride form) was grafted onto HA (Tetra(n-butyl) ammonium or cetyltrimethyl ammonium salt form). The resulting product was obtained as a gel or nanosized colloidal dispersion, and purified by dialysis against sodium EDTA or phosphate buffer-DMSO, and then water and ethanol. Any dialysis system that would remove the ammonium ions is likely to be efficient. Lyophilization of PLA-derivatized HA produced a sponge.

PLA-HA was not soluble in water (although formation of micelles may occur). However, it was soluble in a 1:1 DMSO-water mixture.

In a first aspect the invention relates to a product comprising hyaluronic acid or a salt thereof, wherein the hyaluronic acid or salt thereof is partially or fully linked or crosslinked with a polymer of an alpha hydroxy acid, preferably of poly(lactic acid), also named polylactide, and any lactic acid-based polymers, stereocopolymers and copolymers, especially those with glycolic acid, but also with other co-polymers such as copolymers with hydroxy caproic acid via ϵ -caprolactone, gluconic acid and chemically modified gluconic acid, malic acid, copolymers with low molecular weight segments that can lead to degradation by-products that are hydrosoluble and that can be eliminated via kidney filtration, such as low molecular weight poly(ethylene glycol)s, provided that they bear one or two carboxyl groups at chain ends, and that they provide hydrophobicity in the case of monoacids.

In a second aspect, the invention relates to a composition comprising a product as defined in the first aspect, and an active ingredient, preferably the active ingredient is a pharmacologically active agent.

A third aspect of the invention relates to a pharmaceutical composition comprising an effective amount of a product as defined in the first aspect, together with a pharmaceutically acceptable carrier, excipient or diluent.

A fourth aspect relates to a pharmaceutical composition comprising an effective amount of a product as defined in the first aspect as a vehicle, together with a pharmacologically active agent.

A fifth aspect relates to a cosmetic article comprising as an active ingredient an effective amount of a product as defined in the first aspect.

In a sixth aspect, the invention relates to a sanitary, medical or surgical article comprising a product as defined in the first aspect, preferably the article is a surgical sponge, a wound healing sponge, or a part comprised in a band aid or other wound dressing material.

An important aspect relates to a medicament capsule or microcapsule comprising a product as defined in the first aspect.

Another important aspect of the invention relates to a method of producing a product comprising hyaluronic acid or a salt thereof, wherein the hyaluronic acid is partially or fully linked or crosslinked with a polymer of an alpha hydroxy acid, preferably poly(lactic acid), also named polylactide, and any lactic acid-based polymers, stereocopolymers and copolymers, especially those with glycolic acid, poly(glycolic acid), but also with other co-polymers such as copolymers with hydroxy caproic acid via ϵ -caprolactone, gluconic acid and chemically modified gluconic acid, malic acid, copolymers with low molecular weight

segments that can lead to degradation by-products that are hydrosoluble and that can be eliminated via kidney filtration, such as low molecular weight poly(ethylene glycol)s, provided that they bear one or two carboxyl groups at chain ends, and that they provide hydrophobicity in the case of monoacids, the method comprising the step of:

5 a) reacting hyaluronic acid or a salt thereof with a mono-acyl chloride or di-acyl chloride of the polymer of the alpha hydroxy acid in an organic solvent, preferably in DMSO.

Final aspects of the invention relate to methods of performing procedures in ophtalmology, in the treatment of osteoarthritis or cancer, of treating a wound, of performing dermal or transdermal administration of a pharmacologically active agent to a mammal, or
10 dermal administration of a cosmetic, the improvement which comprises the use of a product as defined in the first aspect, or a composition as defined in any of the second, third, or fourth aspects.

A number of aspects relate to uses of a product as defined in the first aspect or a composition as defined in any of the preceding aspects, for the manufacture of a
15 medicament for the treatment of osteoarthritis, cancer, the manufacture of a medicament for an ophtalmological treatment, the manufacture of a medicament for the treatment of a wound, the manufacture of a medicament for angiogenesis, or the manufacture of a moisturizer.

20 DEFINITIONS

Nucleic Acid Constructs

"Nucleic acid construct" is defined herein as a nucleic acid molecule, either single- or double-stranded, which is isolated from a naturally occurring gene or which has been modified to contain segments of nucleic acid which are combined and juxtaposed in a
25 manner which would not otherwise exist in nature. The term nucleic acid construct may be synonymous with the term expression cassette when the nucleic acid construct contains all the control sequences required for expression of a coding sequence. The term "coding sequence" is defined herein as a sequence which is transcribed into mRNA and translated into an enzyme of interest when placed under the control of the below mentioned control
30 sequences. The boundaries of the coding sequence are generally determined by a ribosome binding site located just upstream of the open reading frame at the 5' end of the mRNA and a transcription terminator sequence located just downstream of the open reading frame at the 3' end of the mRNA. A coding sequence can include, but is not limited to, DNA, cDNA, and recombinant nucleic acid sequences.

35 The techniques used to isolate or clone a nucleic acid sequence encoding a polypeptide are well known in the art and include, for example, isolation from genomic DNA, preparation from cDNA, or a combination thereof. The cloning of the nucleic acid sequences from such genomic DNA can be effected, e.g., by using antibody screening of expression

libraries to detect cloned DNA fragments with shared structural features or the well known polymerase chain reaction (PCR). See, for example, Innis et al., 1990, PCR Protocols: A Guide to Methods and Application, Academic Press, New York. Other nucleic acid amplification procedures such as ligase chain reaction, ligated activated transcription, and
5 nucleic acid sequence-based amplification may be used. The cloning procedures may involve excision and isolation of a desired nucleic acid fragment comprising the nucleic acid sequence encoding the polypeptide, insertion of the fragment into a vector molecule, and incorporation of the recombinant vector into a *Bacillus* cell where clones of the nucleic acid sequence will be replicated. The nucleic acid sequence may be of genomic, cDNA, RNA,
10 semi-synthetic, synthetic origin, or any combinations thereof.

An isolated nucleic acid sequence encoding an enzyme may be manipulated in a variety of ways to provide for expression of the enzyme. Manipulation of the nucleic acid sequence prior to its insertion into a construct or vector may be desirable or necessary depending on the expression vector or *Bacillus* host cell. The techniques for modifying
15 nucleic acid sequences utilizing cloning methods are well known in the art. It will be understood that the nucleic acid sequence may also be manipulated in vivo in the host cell using methods well known in the art.

A number of enzymes are involved in the biosynthesis of hyaluronic acid. These enzymes include hyaluronan synthase, UDP-glucose 6-dehydrogenase, UDP-glucose pyrophosphorylase, UDP-N-acetylglucosamine pyrophosphorylase, glucose-6-phosphate
20 isomerase, hexokinase, phosphoglucomutase, amidotransferase, mutase, and acetyl transferase. Hyaluronan synthase is the key enzyme in the production of hyaluronic acid.

"Hyaluronan synthase" is defined herein as a synthase that catalyzes the elongation of a hyaluronan chain by the addition of GlcUA and GlcNAc sugar precursors. The amino
25 acid sequences of streptococcal hyaluronan synthases, vertebrate hyaluronan synthases, and the viral hyaluronan synthase are distinct from the *Pasteurella* hyaluronan synthase, and have been proposed for classification as Group I and Group II hyaluronan synthases, the Group I hyaluronan synthases including Streptococcal hyaluronan synthases (DeAngelis, 1999). For production of hyaluronan in *Bacillus* host cells, hyaluronan
30 synthases of a eukaryotic origin, such as mammalian hyaluronan synthases, are less preferred.

The hyaluronan synthase encoding sequence may be any nucleic acid sequence capable of being expressed in a *Bacillus* host cell. The nucleic acid sequence may be of any origin. Preferred hyaluronan synthase genes include any of either Group I or Group II,
35 such as the Group I hyaluronan synthase genes from *Streptococcus equisimilis*, *Streptococcus pyogenes*, *Streptococcus uberis*, and *Streptococcus equi* subsp. *zooepidemicus*, or the Group II hyaluronan synthase genes of *Pasturella multocida*.

Constructs whereby precursor sugars of hyaluronan are supplied to the host cell are preferably in producing the HA of the invention, either to the culture medium, or by being encoded by endogenous genes, by non-endogenous genes, or by a combination of endogenous and non-endogenous genes in the *Bacillus* host cell. The precursor sugar may be D-glucuronic acid or N-acetyl-glucosamine.

In the methods of the present invention, the nucleic acid construct may further comprise one or more genes encoding enzymes in the biosynthesis of a precursor sugar of a hyaluronan. Alternatively, the *Bacillus* host cell may further comprise one or more second nucleic acid constructs comprising one or more genes encoding enzymes in the biosynthesis of the precursor sugar. Hyaluronan production is improved by the use of constructs with a nucleic acid sequence or sequences encoding a gene or genes directing a step in the synthesis pathway of the precursor sugar of hyaluronan. By "directing a step in the synthesis pathway of a precursor sugar of hyaluronan" is meant that the expressed protein of the gene is active in the formation of N-acetyl-glucosamine or D-glucuronic acid, or a sugar that is a precursor of either of N-acetyl-glucosamine and D-glucuronic acid.

In a preferred method for supplying precursor sugars, constructs are provided for improving hyaluronan production in a host cell having a hyaluronan synthase, by culturing a host cell having a recombinant construct with a heterologous promoter region operably linked to a nucleic acid sequence encoding a gene directing a step in the synthesis pathway of a precursor sugar of hyaluronan. In a preferred method the host cell also comprises a recombinant construct having a promoter region operably linked to a hyaluronan synthase, which may use the same or a different promoter region than the nucleic acid sequence to a synthase involved in the biosynthesis of N-acetyl-glucosamine. In a further preferred embodiment, the host cell may have a recombinant construct with a promoter region operably linked to different nucleic acid sequences encoding a second gene involved in the synthesis of a precursor sugar of hyaluronan.

Thus, the present invention also relates to constructs for improving hyaluronan production by the use of constructs with a nucleic acid sequence encoding a gene directing a step in the synthesis pathway of a precursor sugar of hyaluronan. The nucleic acid sequence to the precursor sugar may be expressed from the same or a different promoter as the nucleic acid sequence encoding the hyaluronan synthase.

The genes involved in the biosynthesis of precursor sugars for the production of hyaluronic acid include a UDP-glucose 6-dehydrogenase gene, UDP-glucose pyrophosphorylase gene, UDP-N-acetylglucosamine pyrophosphorylase gene, glucose-6-phosphate isomerase gene, hexokinase gene, phosphoglucomutase gene, amidotransferase gene, mutase gene, and acetyl transferase gene.

In a cell containing a hyaluronan synthase, any one or combination of two or more of hasB, hasC and hasD, or the homologs thereof, such as the *Bacillus subtilis* tuaD, gtaB,

and *gcaD*, respectively, as well as *hasE*, may be expressed to increase the pools of precursor sugars available to the hyaluronan synthase. The *Bacillus subtilis* genome is described in Kunst, et al., Nature 390, 249-256, "The complete genome sequence of the Gram-positive bacterium *Bacillus subtilis*" (20 November 1997). In some instances, such as
5 where the host cell does not have a native hyaluronan synthase activity, the construct may include the *hasA* gene.

The nucleic acid sequence encoding the biosynthetic enzymes may be native to the host cell, while in other cases heterologous sequence may be utilized. If two or more genes are expressed they may be genes that are associated with one another in a native operon,
10 such as the genes of the HAS operon of *Streptococcus equisimilis*, which comprises *hasA*, *hasB*, *hasC* and *hasD*. In other instances, the use of some combination of the precursor gene sequences may be desired, without each element of the operon included. The use of some genes native to the host cell, and others which are exogenous may also be preferred in other cases. The choice will depend on the available pools of sugars in a given host cell,
15 the ability of the cell to accommodate overproduction without interfering with other functions of the host cell, and whether the cell regulates expression from its native genes differently than exogenous genes.

As one example, depending on the metabolic requirements and growth conditions of the cell, and the available precursor sugar pools, it may be desirable to increase the
20 production of N-acetyl-glucosamine by expression of a nucleic acid sequence encoding UDP-N-acetylglucosamine pyrophosphorylase, such as the *hasD* gene, the *Bacillus gcaD* gene, and homologs thereof. Alternatively, the precursor sugar may be D-glucuronic acid. In one such embodiment, the nucleic acid sequence encodes UDP-glucose 6-dehydrogenase. Such nucleic acid sequences include the *Bacillus tuaD* gene, the *hasB*
25 gene of *Streptococcus*, and homologs thereof. The nucleic acid sequence may also encode UDP-glucose pyrophosphorylase, such as in the *Bacillus gtaB* gene, the *hasC* gene of *Streptococcus*, and homologues thereof. In the methods of the present invention, the UDP-glucose 6-dehydrogenase gene may be a *hasB* gene or *tuaD* gene; or homologues thereof.

In the present invention it is envisioned that the hyaluronan synthase gene and the
30 one or more genes encoding a precursor sugar are under the control of the same promoter. Alternatively, the one or more genes encoding a precursor sugar are under the control of the same promoter but a different promoter driving the hyaluronan synthase gene. A further alternative is that the hyaluronan synthase gene and each of the genes encoding a
35 precursor sugar are under the control of different promoters. In a preferred embodiment, the hyaluronan synthase gene and the one or more genes encoding a precursor sugar are under the control of the same promoter.

The present invention also relates to a nucleic acid construct comprising an isolated nucleic acid sequence encoding a hyaluronan synthase operon comprising a hyaluronan synthase gene and a UDP-glucose 6-dehydrogenase gene, and optionally one or more genes selected from the group consisting of a UDP-glucose pyrophosphorylase gene, UDP-
5 N-acetylglucosamine pyrophosphorylase gene, and glucose-6-phosphate isomerase gene.

In some cases the host cell will have a recombinant construct with a heterologous promoter region operably linked to a nucleic acid sequence encoding a gene directing a step in the synthesis pathway of a precursor sugar of hyaluronan, which may be in concert with the expression of hyaluronan synthase from a recombinant construct. The hyaluronan
10 synthase may be expressed from the same or a different promoter region than the nucleic acid sequence encoding an enzyme involved in the biosynthesis of the precursor. In another preferred embodiment, the host cell may have a recombinant construct with a promoter region operably linked to a different nucleic acid sequence encoding a second gene involved in the synthesis of a precursor sugar of hyaluronan.

The nucleic acid sequence encoding the enzymes involved in the biosynthesis of the precursor sugar(s) may be expressed from the same or a different promoter as the nucleic acid sequence encoding the hyaluronan synthase. In the former sense, "artificial operons" are constructed, which may mimic the operon of *Streptococcus equisimilis* in having each hasA, hasB, hasC and hasD, or homologs thereof, or, alternatively, may utilize
20 less than the full complement present in the *Streptococcus equisimilis* operon. The artificial operons" may also comprise a glucose-6-phosphate isomerase gene (hasE) as well as one or more genes selected from the group consisting of a hexokinase gene, phosphoglucomutase gene, amidotransferase gene, mutase gene, and acetyl transferase gene. In the artificial operon, at least one of the elements is heterologous to one other of the
25 elements, such as the promoter region being heterologous to the encoding sequences.

In a preferred embodiment, the nucleic acid construct comprises hasA, tuaD, and gtaB. In another preferred embodiment, the nucleic acid construct comprises hasA, tuaD, gtaB, and gcaD. In another preferred embodiment, the nucleic acid construct comprises hasA and tuaD. In another preferred embodiment, the nucleic acid construct comprises
30 hasA. In another preferred embodiment, the nucleic acid construct comprises hasA, tuaD, gtaB, gcaD, and hasE. In another preferred embodiment, the nucleic acid construct comprises hasA, hasB, hasC, and hasD. In another preferred embodiment, the nucleic acid construct comprises hasA, hasB, hasC, hasD, and hasE. Based on the above preferred embodiments, the genes noted can be replaced with homologs thereof.

In the methods of the present invention, the nucleic acid constructs comprise a
35 hyaluronan synthase encoding sequence operably linked to a promoter sequence foreign to the hyaluronan synthase encoding sequence. The promoter sequence may be, for example, a single promoter or a tandem promoter.

"Promoter" is defined herein as a nucleic acid sequence involved in the binding of RNA polymerase to initiate transcription of a gene. "Tandem promoter" is defined herein as two or more promoter sequences each of which is operably linked to a coding sequence and mediates the transcription of the coding sequence into mRNA. "Operably linked" is defined
5 herein as a configuration in which a control sequence, e.g., a promoter sequence, is appropriately placed at a position relative to a coding sequence such that the control sequence directs the production of a polypeptide encoded by the coding sequence. As noted earlier, a "coding sequence" is defined herein as a nucleic acid sequence which is transcribed into mRNA and translated into a polypeptide when placed under the control of
10 the appropriate control sequences. The boundaries of the coding sequence are generally determined by a ribosome binding site located just upstream of the open reading frame at the 5' end of the mRNA and a transcription terminator sequence located just downstream of the open reading frame at the 3' end of the mRNA. A coding sequence can include, but is not limited to, genomic DNA, cDNA, semisynthetic, synthetic, and recombinant nucleic acid
15 sequences.

In a preferred embodiment, the promoter sequences may be obtained from a bacterial source. In a more preferred embodiment, the promoter sequences may be obtained from a gram positive bacterium such as a *Bacillus* strain, e.g., *Bacillus agaradherens*, *Bacillus alkalophilus*, *Bacillus amyloliquefaciens*, *Bacillus brevis*, *Bacillus circulans*, *Bacillus clausii*, *Bacillus coagulans*, *Bacillus firmus*, *Bacillus lautus*, *Bacillus lentus*, *Bacillus licheniformis*, *Bacillus megaterium*, *Bacillus pumilus*, *Bacillus stearothermophilus*, *Bacillus subtilis*, or *Bacillus thuringiensis*; or a *Streptomyces* strain, e.g., *Streptomyces lividans* or *Streptomyces murinus*; or from a gram negative bacterium, e.g., *E. coli* or *Pseudomonas sp.*

25 Examples of suitable promoters for directing the transcription of a nucleic acid sequence in the methods of the present invention are the promoters obtained from the *E. coli* lac operon, *Streptomyces coelicolor* agarase gene (dagA), *Bacillus lentus* or *Bacillus clausii* alkaline protease gene (aprH), *Bacillus licheniformis* alkaline protease gene (subtilisin Carlsberg gene), *Bacillus subtilis* levansucrase gene (sacB), *Bacillus subtilis*
30 alpha-amylase gene (amyE), *Bacillus licheniformis* alpha-amylase gene (amyL), *Bacillus stearothermophilus* maltogenic amylase gene (amyM), *Bacillus amyloliquefaciens* alpha-amylase gene (amyQ), *Bacillus licheniformis* penicillinase gene (penP), *Bacillus subtilis* xylA and xylB genes, *Bacillus thuringiensis* subsp. *tenebrionis* CryIIIA gene (cryIIIA) or portions thereof, prokaryotic beta-lactamase gene (Villa-Kamaroff et al., 1978, Proceedings of the
35 National Academy of Sciences USA 75:3727-3731). Other examples are the promoter of the spo1 bacterial phage promoter and the tac promoter (DeBoer et al., 1983, Proceedings of the National Academy of Sciences USA 80:21-25). Further promoters are described in "Useful proteins from recombinant bacteria" in Scientific American, 1980, 242:74-94; and in

Sambrook, Fritsch, and Maniatus, 1989, Molecular Cloning, A Laboratory Manual, 2d edition, Cold Spring Harbor, New York.

The promoter may also be a "consensus" promoter having the sequence TTGACA for the "-35" region and TATAAT for the "-10" region. The consensus promoter may be obtained from any promoter which can function in a *Bacillus* host cell. The construction of a "consensus" promoter may be accomplished by site-directed mutagenesis to create a promoter which conforms more perfectly to the established consensus sequences for the "10" and "-35" regions of the vegetative "sigma A-type" promoters for *Bacillus subtilis* (Voskuil et al., 1995, Molecular Microbiology 17: 271-279).

In a preferred embodiment, the "consensus" promoter is obtained from a promoter obtained from the *E. coli* lac operon, *Streptomyces coelicolor* agarase gene (*dagA*), *Bacillus clausii* or *Bacillus lentus* alkaline protease gene (*aprH*), *Bacillus licheniformis* alkaline protease gene (*subtilisin Carlsberg* gene), *Bacillus subtilis* levansucrase gene (*sacB*), *Bacillus subtilis* alpha-amylase gene (*amyE*), *Bacillus licheniformis* alpha-amylase gene (*amyL*), *Bacillus stearothermophilus* maltogenic amylase gene (*amyM*), *Bacillus amyloliquefaciens* alpha-amylase gene (*amyQ*), *Bacillus licheniformis* penicillinase gene (*penP*), *Bacillus subtilis* *xylA* and *xylB* genes, *Bacillus thuringiensis* subsp. *tenebrionis* *CryIIIA* gene (*cryIIIA*) or portions thereof, or prokaryotic beta-lactamase gene *spo1* bacterial phage promoter. In a more preferred embodiment, the "consensus" promoter is obtained from *Bacillus amyloliquefaciens* alpha-amylase gene (*amyQ*).

Widner, et al., United States Patent Nos. 6,255,076 and 5,955,310, describe tandem promoters and constructs and methods for use in expression in *Bacillus* cells, including the short consensus *amyQ* promoter (also called *scBAN*). The use of the *cryIIIA* stabilizer sequence, and constructs using the sequence, for improved production in *Bacillus* are also described therein.

Each promoter sequence of the tandem promoter may be any nucleic acid sequence which shows transcriptional activity in the *Bacillus* cell of choice including a mutant, truncated, and hybrid promoter, and may be obtained from genes encoding extracellular or intracellular polypeptides either homologous or heterologous to the *Bacillus* cell. Each promoter sequence may be native or foreign to the nucleic acid sequence encoding the polypeptide and native or foreign to the *Bacillus* cell. The promoter sequences may be the same promoter sequence or different promoter sequences.

The two or more promoter sequences of the tandem promoter may simultaneously promote the transcription of the nucleic acid sequence. Alternatively, one or more of the promoter sequences of the tandem promoter may promote the transcription of the nucleic acid sequence at different stages of growth of the *Bacillus* cell.

In a preferred embodiment, the tandem promoter contains at least the *amyQ* promoter of the *Bacillus amyloliquefaciens* alpha-amylase gene. In another preferred

embodiment, the tandem promoter contains at least a "consensus" promoter having the sequence TTGACA for the "-35" region and TATAAT for the "-10" region. In another preferred embodiment, the tandem promoter contains at least the amyL promoter of the *Bacillus licheniformis* alpha-amylase gene. In another preferred embodiment, the tandem promoter contains at least the cryIIIA promoter or portions thereof (Agaisse and Lereclus, 1994, *Molecular Microbiology* 13: 97-107).

In a more preferred embodiment, the tandem promoter contains at least the amyL promoter and the cryIIIA promoter. In another more preferred embodiment, the tandem promoter contains at least the amyQ promoter and the cryIIIA promoter. In another more preferred embodiment, the tandem promoter contains at least a "consensus" promoter having the sequence TTGACA for the "-35" region and TATAAT for the "-10" region and the cryIIIA promoter. In another more preferred embodiment, the tandem promoter contains at least two copies of the amyL promoter. In another more preferred embodiment, the tandem promoter contains at least two copies of the amyQ promoter. In another more preferred embodiment, the tandem promoter contains at least two copies of a "consensus" promoter having the sequence TTGACA for the "-35" region and TATAAT for the "-10" region. In another more preferred embodiment, the tandem promoter contains at least two copies of the cryIIIA promoter.

"An mRNA processing/stabilizing sequence" is defined herein as a sequence located downstream of one or more promoter sequences and upstream of a coding sequence to which each of the one or more promoter sequences are operably linked such that all mRNAs synthesized from each promoter sequence may be processed to generate mRNA transcripts with a stabilizer sequence at the 5' end of the transcripts. The presence of such a stabilizer sequence at the 5' end of the mRNA transcripts increases their half-life (Agaisse and Lereclus, 1994, *supra*, Hue et al., 1995, *Journal of Bacteriology* 177: 3465-3471). The mRNA processing/stabilizing sequence is complementary to the 3' extremity of a bacterial 16S ribosomal RNA. In a preferred embodiment, the mRNA processing/stabilizing sequence generates essentially single-size transcripts with a stabilizing sequence at the 5' end of the transcripts. The mRNA processing/stabilizing sequence is preferably one, which is complementary to the 3' extremity of a bacterial 16S ribosomal RNA. See, U.S. Patent Nos. 6,255,076 and 5,955,310.

In a more preferred embodiment, the mRNA processing/stabilizing sequence is the *Bacillus thuringiensis* cryIIIA mRNA processing/stabilizing sequence disclosed in WO 94/25612 and Agaisse and Lereclus, 1994, *supra*, or portions thereof which retain the mRNA processing/stabilizing function. In another more preferred embodiment, the mRNA processing/stabilizing sequence is the *Bacillus subtilis* SP82 mRNA processing/stabilizing sequence disclosed in Hue et al., 1995, *supra*, or portions thereof which retain the mRNA processing/stabilizing function.

When the cryIIIA promoter and its mRNA processing/stabilizing sequence are employed in the methods of the present invention, a DNA fragment containing the sequence disclosed in WO 94/25612 and Agaisse and Lereclus, 1994, supra, or portions thereof which retain the promoter and mRNA processing/stabilizing functions, may be used. Furthermore, DNA fragments containing only the cryIIIA promoter or only the cryIIIA mRNA processing/stabilizing sequence may be prepared using methods well known in the art to construct various tandem promoter and mRNA processing/stabilizing sequence combinations. In this embodiment, the cryIIIA promoter and its mRNA processing/stabilizing sequence are preferably placed downstream of the other promoter sequence(s) constituting the tandem promoter and upstream of the coding sequence of the gene of interest.

The isolated nucleic acid sequence encoding the desired enzyme(s) involved in hyaluronic acid production may then be further manipulated to improve expression of the nucleic acid sequence. Expression will be understood to include any step involved in the production of the polypeptide including, but not limited to, transcription, post-transcriptional modification, translation, post-translational modification, and secretion. The techniques for modifying nucleic acid sequences utilizing cloning methods are well known in the art.

A nucleic acid construct comprising a nucleic acid sequence encoding an enzyme may be operably linked to one or more control sequences capable of directing the expression of the coding sequence in a *Bacillus* cell under conditions compatible with the control sequences.

The term "control sequences" is defined herein to include all components which are necessary or advantageous for expression of the coding sequence of a nucleic acid sequence. Each control sequence may be native or foreign to the nucleic acid sequence encoding the enzyme. In addition to promoter sequences described above, such control sequences include, but are not limited to, a leader, a signal sequence, and a transcription terminator. At a minimum, the control sequences include a promoter, and transcriptional and translational stop signals. The control sequences may be provided with linkers for the purpose of introducing specific restriction sites facilitating ligation of the control sequences with the coding region of the nucleic acid sequence encoding an enzyme.

The control sequence may also be a suitable transcription terminator sequence, a sequence recognized by a *Bacillus* cell to terminate transcription. The terminator sequence is operably linked to the 3' terminus of the nucleic acid sequence encoding the enzyme or the last enzyme of an operon. Any terminator which is functional in the *Bacillus* cell of choice may be used in the present invention.

The control sequence may also be a suitable leader sequence, a nontranslated region of a mRNA which is important for translation by the *Bacillus* cell. The leader sequence is operably linked to the 5' terminus of the nucleic acid sequence encoding the

enzyme. Any leader sequence which is functional in the *Bacillus* cell of choice may be used in the present invention.

The control sequence may also be a signal peptide coding region, which codes for an amino acid sequence linked to the amino terminus of a polypeptide which can direct the expressed polypeptide into the cell's secretory pathway. The signal peptide coding region may be native to the polypeptide or may be obtained from foreign sources. The 5' end of the coding sequence of the nucleic acid sequence may inherently contain a signal peptide coding region naturally linked in translation reading frame with the segment of the coding region which encodes the secreted polypeptide. Alternatively, the 5' end of the coding sequence may contain a signal peptide coding region which is foreign to that portion of the coding sequence which encodes the secreted polypeptide. The foreign signal peptide coding region may be required where the coding sequence does not normally contain a signal peptide coding region. Alternatively, the foreign signal peptide coding region may simply replace the natural signal peptide coding region in order to obtain enhanced secretion of the polypeptide relative to the natural signal peptide coding region normally associated with the coding sequence. The signal peptide coding region may be obtained from an amylase or a protease gene from a *Bacillus* species. However, any signal peptide coding region capable of directing the expressed polypeptide into the secretory pathway of a *Bacillus* cell of choice may be used in the present invention.

An effective signal peptide coding region for *Bacillus* cells is the signal peptide coding region obtained from the maltogenic amylase gene from *Bacillus* NCIB 11837, the *Bacillus stearothermophilus* alpha-amylase gene, the *Bacillus licheniformis* subtilisin gene, the *Bacillus licheniformis* beta-lactamase gene, the *Bacillus stearothermophilus* neutral proteases genes (nprT, nprS, nprM), and the *Bacillus subtilis* prsA gene. Further signal peptides are described by Simonen and Palva, 1993, *Microbiological Reviews* 57:109-137.

The control sequence may also be a propeptide coding region that codes for an amino acid sequence positioned at the amino terminus of a polypeptide. The resultant polypeptide is known as a proenzyme or propolypeptide (or a zymogen in some cases). A propolypeptide is generally inactive and can be converted to a mature active polypeptide by catalytic or autocatalytic cleavage of the propeptide from the propolypeptide. The propeptide coding region may be obtained from the genes for *Bacillus subtilis* alkaline protease (aprE) and *Bacillus subtilis* neutral protease (nprT).

Where both signal peptide and propeptide regions are present at the amino terminus of a polypeptide, the propeptide region is positioned next to the amino terminus of a polypeptide and the signal peptide region is positioned next to the amino terminus of the propeptide region.

It may also be desirable to add regulatory sequences which allow the regulation of the expression of the polypeptide relative to the growth of the host cell. Examples of

regulatory systems are those which cause the expression of the gene to be turned on or off in response to a chemical or physical stimulus, including the presence of a regulatory compound. Regulatory systems in prokaryotic systems include the lac, tac, and trp operator systems.

5

Production

In the methods of the present invention, the host cells are cultivated in a nutrient medium suitable for production of the hyaluronic acid using methods known in the art. For example, the cell may be cultivated by shake flask cultivation, small-scale or large-scale
10 fermentation (including continuous, batch, fed-batch, or solid state fermentations) in laboratory or industrial fermentors performed in a suitable medium and under conditions allowing the enzymes involved in hyaluronic acid synthesis to be expressed and the hyaluronic acid to be isolated. The cultivation takes place in a suitable nutrient medium comprising carbon and nitrogen sources and inorganic salts, using procedures known in the
15 art. Suitable media are available from commercial suppliers or may be prepared according to published compositions (e.g., in catalogues of the American Type Culture Collection). The secreted hyaluronic acid can be recovered directly from the medium.

The resulting hyaluronic acid may be isolated by methods known in the art. For example, the hyaluronic acid may be isolated from the nutrient medium by conventional
20 procedures including, but not limited to, centrifugation, filtration, extraction, spraydrying, evaporation, or precipitation. The isolated hyaluronic acid may then be further purified by a variety of procedures known in the art including, but not limited to, chromatography (e.g., ion exchange, affinity, hydrophobic, chromatofocusing, and size exclusion), electrophoretic procedures (e.g., preparative isoelectric focusing), differential solubility (e.g., ammonium
25 sulfate precipitation), or extraction (see, e.g., Protein Purification, J.-C. Janson and Lars Ryden, editors, VCH Publishers, New York, 1989).

FIGURES

Figure 1 shows structural formulae of various compounds used herein.

30 Figure 2 shows the reaction scheme of poly(lactic acid) with thionyl chloride to form PLA acyl-chloride.

Figure 3 shows the IR spectrum of poly(lactic acid).

Figure 4 shows the IR spectrum of hyaluronic acid (proton form).

Figure 5 shows the IR spectrum of the final synthesis product in example 3, HA-PLA.

35 Figure 6 shows the ¹³C spectrum of the final synthesis product in example 3, HA-PLA.

Figure 7 shows the reaction scheme of HA-TBA with PLA di-acyl-chloride to form HA-PLA-HA as outlined in example 4.

Figure 8 shows the IR spectrum of the ethanol-wash in example 4, with probable HA-PLA-HA present.

Figure 9 shows the IR spectrum of the acetone-wash in example 4, with probable HA-PLA-HA present.

5 Figure 10 shows the IR spectrum of the final product from HA-CTA in example 4; HA-PLA-HA.

Figure 11 shows the IR spectrum of the final product from HA-TBA in example 4; HA-PLA-HA.

Figure 12 shows the IR spectrum of the HA-CTA synthesized in example 5.

10 Figure 13 shows the ¹H NMR of the final HA-PLA product of example 6, the spectrum shows the presence of remnant CTA.

Figure 14 shows the ¹H NMR of the final HA-PLA product of example 6, after the dialysis of example 7.

15 DETAILED DESCRIPTION OF THE INVENTION

"Hyaluronic acid" is defined herein as an unsulphated glycosaminoglycan composed of repeating disaccharide units of N-acetylglucosamine (GlcNAc) and glucuronic acid (GlcUA) linked together by alternating beta-1,4 and beta-1,3 glycosidic bonds. Hyaluronic acid is also known as hyaluronan, hyaluronate, or HA. The terms hyaluronan and hyaluronic acid are used interchangeably herein.

20 Rooster combs are a significant commercial source for hyaluronan. Microorganisms are an alternative source. U.S. Patent No. 4,801,539 discloses a fermentation method for preparing hyaluronic acid involving a strain of *Streptococcus zooepidemicus* with reported yields of about 3.6 g of hyaluronic acid per liter. European Patent No. EP0694616 discloses fermentation processes using an improved strain of *Streptococcus zooepidemicus* with reported yields of about 3.5 g of hyaluronic acid per liter. As disclosed in WO 03/054163 (Novozymes), which is incorporated herein in its entirety, hyaluronic acid or salts thereof may be recombinantly produced, e.g., in a Gram-positive *Bacillus* host.

30 Hyaluronan synthases have been described from vertebrates, bacterial pathogens, and algal viruses (DeAngelis, P. L., 1999, *Cell. Mol. Life Sci.* 56: 670-682). WO 99/23227 discloses a Group I hyaluronate synthase from *Streptococcus equisimilis*. WO 99/51265 and WO 00/27437 describe a Group II hyaluronate synthase from *Pasturella multocida*. Ferretti et al. disclose the hyaluronan synthase operon of *Streptococcus pyogenes*, which is composed of three genes, *hasA*, *hasB*, and *hasC*, that encode hyaluronate synthase, UDP glucose dehydrogenase, and UDP-glucose pyrophosphorylase, respectively (Proc. Natl. Acad. Sci. USA. 98, 4658-4663, 2001). WO 99/51265 describes a nucleic acid segment having a coding region for a *Streptococcus equisimilis* hyaluronan synthase.

Since the hyaluronan of a recombinant *Bacillus* cell is expressed directly to the culture medium, a simple process may be used to isolate the hyaluronan from the culture medium. First, the *Bacillus* cells and cellular debris are physically removed from the culture medium. The culture medium may be diluted first, if desired, to reduce the viscosity of the medium. Many methods are known to those skilled in the art for removing cells from culture medium, such as centrifugation or microfiltration. If desired, the remaining supernatant may then be filtered, such as by ultrafiltration, to concentrate and remove small molecule contaminants from the hyaluronan. Following removal of the cells and cellular debris, a simple precipitation of the hyaluronan from the medium is performed by known mechanisms. Salt, alcohol, or combinations of salt and alcohol may be used to precipitate the hyaluronan from the filtrate. Once reduced to a precipitate, the hyaluronan can be easily isolated from the solution by physical means. The hyaluronan may be dried or concentrated from the filtrate solution by using evaporative techniques known to the art, such as lyophilization or spraydrying.

The first aspect of the invention relates to a product comprising hyaluronic acid or a salt thereof, wherein the hyaluronic acid has been partially or fully linked or crosslinked with a polymer of an alpha hydroxy acid, preferably of poly(lactic acid), also named polylactide, and any lactic acid-based polymers, stereocopolymers and copolymers, especially those with glycolic acid, but also with other co-polymers such as copolymers with hydroxy caproic acid via ϵ -caprolactone, gluconic acid and chemically modified gluconic acid, malic acid, copolymers with low molecular weight segments that can lead to degradation by-products that are hydrosoluble and that can be eliminated via kidney filtration, such as low molecular weight poly(ethylene glycol)s, provided that they bear one or two carboxyl groups at chain ends, and that they provide hydrophobicity in the case of monoacids.

Host Cells

A preferred embodiment relates to the product of the first aspect, wherein the hyaluronic acid or salt thereof is recombinantly produced, preferably by a Gram-positive bacterium or host cell, more preferably by a bacterium of the genus *Bacillus*.

The host cell may be any *Bacillus* cell suitable for recombinant production of hyaluronic acid. The *Bacillus* host cell may be a wild-type *Bacillus* cell or a mutant thereof. *Bacillus* cells useful in the practice of the present invention include, but are not limited to, *Bacillus agaraderhens*, *Bacillus alkalophilus*, *Bacillus amyloliquefaciens*, *Bacillus brevis*, *Bacillus circulans*, *Bacillus clausii*, *Bacillus coagulans*, *Bacillus firmus*, *Bacillus lautus*, *Bacillus lentus*, *Bacillus licheniformis*, *Bacillus megaterium*, *Bacillus pumilus*, *Bacillus stearothermophilus*, *Bacillus subtilis*, and *Bacillus thuringiensis* cells. Mutant *Bacillus subtilis*

cells particularly adapted for recombinant expression are described in WO 98/22598. Non-encapsulating *Bacillus* cells are particularly useful in the present invention.

In a preferred embodiment, the *Bacillus* host cell is a *Bacillus amyloliquefaciens*, *Bacillus clausii*, *Bacillus lentus*, *Bacillus licheniformis*, *Bacillus stearothermophilus* or *Bacillus subtilis* cell. In a more preferred embodiment, the *Bacillus* cell is a *Bacillus amyloliquefaciens* cell. In another more preferred embodiment, the *Bacillus* cell is a *Bacillus clausii* cell. In another more preferred embodiment, the *Bacillus* cell is a *Bacillus lentus* cell. In another more preferred embodiment, the *Bacillus* cell is a *Bacillus licheniformis* cell. In another more preferred embodiment, the *Bacillus* cell is a *Bacillus subtilis* cell. In a most preferred embodiment, the *Bacillus* host cell is *Bacillus subtilis* A164Δ5 (see U.S. Patent No. 5,891,701) or *Bacillus subtilis* 168Δ4.

Transformation of the *Bacillus* host cell with a nucleic acid construct of the present invention may, for instance, be effected by protoplast transformation (see, e.g., Chang and Cohen, 1979, *Molecular General Genetics* 168: 111-115), by using competent cells (see, e.g., Young and Spizizen, 1961, *Journal of Bacteriology* 81: 823-829, or Dubnau and Davidoff-Abelson, 1971, *Journal of Molecular Biology* 56: 209-221), by electroporation (see, e.g., Shigekawa and Dower, 1988, *Biotechniques* 6: 742-751), or by conjugation (see, e.g., Koehler and Thorne, 1987, *Journal of Bacteriology* 169: 5271-5278).

Molecular weight

The level of hyaluronic acid may be determined according to the modified carbazole method (Bitter and Muir, 1962, *Anal Biochem.* 4: 330-334). Moreover, the average molecular weight of the hyaluronic acid may be determined using standard methods in the art, such as those described by Ueno *et al.*, 1988, *Chem. Pharm. Bull.* 36, 4971-4975; Wyatt, 1993, *Anal. Chim. Acta* 272: 1-40; and Wyatt Technologies, 1999, "Light Scattering University DAWN Course Manual" and "DAWN EOS Manual" Wyatt Technology Corporation, Santa Barbara, California.

In a preferred embodiment, the hyaluronic acid obtained by the methods of the present invention has a molecular weight of about 10,000 to about 10,000,000 Da. In a more preferred embodiment, the hyaluronic acid obtained by the methods of the present invention has a molecular weight of about 25,000 to about 5,000,000 Da. In a most preferred embodiment, the hyaluronic acid obtained by the methods of the present invention has a molecular weight of about 50,000 to about 3,000,000 Da.

A preferred embodiment relates to the product of the first aspect, wherein the hyaluronic acid or salt thereof has a molecular weight in the range of between 300,000 and 3,000,000; preferably in the range of between 400,000 and 2,500,000; more preferably in the range of between 500,000 and 2,000,000; and most preferably in the range of between 600,000 and 1,800,000.

Salts and crosslinked HA

A preferred embodiment relates to a product of the first aspect, which comprises an inorganic salt of hyaluronic acid, preferably sodium hyaluronate, potassium hyaluronate, ammonium hyaluronate, calcium hyaluronate, magnesium hyaluronate, zinc hyaluronate, or cobalt hyaluronate.

In the examples below it was found that the reaction of sodium hyaluronate with poly(lactic acid) mono- or di-acyl chloride resulted in a linked or crosslinked HA-PLA or HA-PLA-HA product, which showed an intensified peak at 1736 cm⁻¹ on the IR spectrum, when compared to a standard spectrum of the untreated HA or PLA, corresponding to the presence of newly linked poly(lactic acid) segments to HA to form HA-PLA product.

Accordingly, a preferred embodiment relates to the product of the first aspect, wherein the crosslinked hyaluronic acid or salt thereof comprises esters of a polymeric alpha hydroxy acid, preferably of poly(lactic acid), also named polylactide, and any lactic acid-based polymers, stereocopolymers and copolymers, especially those with glycolic acid, but also with other co-polymers such as copolymers with hydroxy caproic acid via ϵ -caprolactone, gluconic acid and chemically modified gluconic acid, malic acid, copolymers with low molecular weight segments that can lead to degradation by-products that are hydrosoluble and that can be eliminated via kidney filtration, such as low molecular weight poly(ethylene glycol)s, provided that they bear one or two carboxyl groups at chain ends, and that they provide hydrophobicity in the case of monoacids.

Determination of moisture

The moisture content of a dried product powder according to the invention is the loss in weight, expressed as a percentage, after drying the powder at 102°C $\pm$ 2°C to a constant weight. An empty glass weighing dish with a ground lid is dried in the oven, then cooled and weighed on an analytical balance with a sensitivity of at least 0.1 mg. Approximately 3 g dried product powder is placed in the dish and weighed. The dish with the powder is placed without the lid in the oven and dried for 2 hours at a temperature of 102°C $\pm$ 2°C; then it is placed in a desiccator and cooled to room temperature before it is weighed again. The dish with the powder is placed without the lid in the oven to dry for 1 more hour, and then cooled and weighed as already described; this is repeated until the weight remains constant, *i.e.*, until two successive weighings do not differ by more than 0.5 mg.

The percentage of moisture is then calculated as: $(W2-W3)/(W2-W1) \times 100$; where W1 is the weight of the empty dish, W2 is the weight of the dish with powder, and W3 is the

weight of the dish with dried powder. The result is calculated to 2 decimal places, and the reproducibility of this method is about $\pm 0.1\%$.

In a preferred embodiment, the product of the first aspect is dried and comprises less than 5% moisture, preferably less than 2%, and most preferably less than 1% moisture, as determined herein.

Other ingredients

In a preferred embodiment, the product of the invention may also comprise other ingredients, preferably one or more active ingredient, preferably one or more pharmacologically active substance, and also preferably a water-soluble excipient, such as lactose.

Non-limiting examples of an active ingredient or pharmacologically active substance which may be used in the present invention include protein and/or peptide drugs, such as, human growth hormone, bovine growth hormone, porcine growth hormone, growth hormone releasing hormone/peptide, granulocyte-colony stimulating factor, granulocyte macrophage-colony stimulating factor, macrophage-colony stimulating factor, erythropoietin, bone morphogenic protein, interferon or derivative thereof, insulin or derivative thereof, atriopeptin-III, monoclonal antibody, tumor necrosis factor, macrophage activating factor, interleukin, tumor degenerating factor, insulin-like growth factor, epidermal growth factor, tissue plasminogen activator, factor IIV, factor IIIV, and urokinase.

A water-soluble excipient may be included for the purpose of stabilizing the active ingredient(s), such excipient may include a protein, e.g., albumin or gelatin; an amino acid, such as glycine, alanine, glutamic acid, arginine, lysine and a salt thereof; carbohydrate such as glucose, lactose, xylose, galactose, fructose, maltose, saccharose, dextran, mannitol, sorbitol, trehalose and chondroitin sulphate; an inorganic salt such as phosphate; a surfactant such as TWEEN® (ICI), poly ethylene glycol, and a mixture thereof. The excipient or stabilizer may be used in an amount ranging from 0.001 to 99% by weight of the product.

Several aspects of the invention relate to various compositions and pharmaceutical comprising, among other constituents, an effective amount of the product as defined in the first aspect, and an active ingredient, preferably the active ingredient is a pharmacologically active agent; a pharmaceutically acceptable carrier, excipient or diluent, preferably a water-soluble excipient, and most preferably lactose.

In addition, aspects of the invention relate to articles comprising a product as defined in the first aspect or a composition as defined in the aspects and embodiments above, e.g., a cosmetic article, a sanitary article, a medical or surgical article. In a final aspect the invention relates to a medicament capsule or microcapsule comprising a product as defined

in the first aspect or a composition as defined in other aspects and embodiments of the invention.

Method of production

5 The present invention in another aspect provides a method of producing a product comprising hyaluronic acid or a salt thereof, wherein the hyaluronic acid is partially or fully linked or crosslinked with a polymer of an alpha hydroxy acid, preferably poly(lactic acid), also named polylactide, and any lactic acid-based polymers, stereocopolymers and copolymers, especially those with glycolic acid, but also with other co-polymers such as
10 copolymers with hydroxy caproic acid via ϵ -caprolactone, gluconic acid and chemically modified gluconic acid, malic acid, copolymers with low molecular weight segments that can lead to degradation by-products that are hydrosoluble and that can be eliminated via kidney filtration, such as low molecular weight poly(ethylene glycol)s, provided that they bear one or two carboxyl groups at chain ends, and that they provide hydrophobicity in the case of
15 monoacids, the method comprising the step of:

- a) reacting hyaluronic acid or a salt thereof with a mono-acyl chloride or di-acyl chloride of the polymer of the alpha hydroxy acid in an organic solvent, preferably in DMSO.

Methods of using the product or composition

20 Various aspects of the invention relate to methods of performing treatment procedures, e.g., in the medical field, using a product of the first aspect, or using compositions of the invention.

 One aspect relates to a method of performing procedures in ophthalmology, which comprises the use of a product as defined in the first aspect or a composition of the
25 invention.

 Another aspect relates to a method of performing procedures in the treatment of osteoarthritis, which comprises the use of a product as defined in the first aspect or a composition of the invention.

 Yet another aspect relates to a method of performing procedures in the treatment of
30 cancer, which comprises the use of a product as defined in the first aspect or a composition of the invention.

 An aspect relates to a method of performing transdermal or dermal administration of a pharmacologically active agent, which comprises the use of a product as defined in the first aspect or a composition of the invention.

35 Another aspect relates to a method of performing dermal administration of a cosmetic, which comprises the use of a product or a composition of the invention.

EXAMPLES

A number of chemical abbreviations are shown by structural formulae in figure 1, e.g. tetrabutyl ammonium (TBA), cetyltrimethyl ammonium (CTA), hyaluronic acid (HA), and poly(lactic acid) (PLA).

5

Example 1. DMSO and SOCl₂ distillations

DMSO distillation

1.5 l of DMSO was introduced in a 2000 ml round bottom flask, and with magnetic stirring a small amount of P₂O₅ was added to the DMSO, in order to withdraw the water.

10 The flask was then set up for vacuum distillation with the condenser fitted to a rotatable multi-receiver adapter with one 100 ml, and two 1000 ml flasks, allowing 3 fractions to be individually collected without having to interrupt the distillation.

The flask was heated to about 75°C under vacuum to distill. The first small fraction was collected in the 100 ml round bottomed flask and later discarded. The distilled DMSO
15 was finally collected. The temperature at the top of the column was 42°C and the vacuum was 2 mbar. Ultrapure commercial DMSO can be used without distillation.

SOCl₂ distillation

300 ml of thionyl chloride, SOCl₂, was introduced in a 500 ml round bottomed flask,
20 and with magnetic stirring 50 ml of triphenylphosphite was added dropwise in order to trap chlorine and sulphur. It is important to control the temperature during the addition as it is very exothermic.

When all the triphenylphosphite had been added, the flask was set up for distillation with the condenser fitted to a rotatable multi-receiver adapter with a 50 ml, a 100 ml, and a
25 250 ml flask, allowing 3 fractions to be individually collected without having to interrupt the distillation. The entire setup was wrapped in an aluminium sheet to protect the distilled SOCl₂ from light. A calciumchloride trap was also fitted to the distillation setup to protect from water. The flask was then heated until 105 °C to distill the product; the temperature at the top of the column was 72 °C.

30

Example 2. Preparation of PLA acyl-chloride

The reaction scheme is shown in figure 2. 2,4454 g of PLA was dissolved in freshly distilled SOCl₂ and introduced in a round bottomed flask of 250 ml with magnetic stirring, which was then set up for reflux and protected from water by a CaCl₂ trap. The flask was
35 heated to reflux (about 80 °C) for 3 hours.

When the reaction was finished, SOCl₂ in excess was distilled off at 60 °C under vacuum. To remove all the SOCl₂, the remaining product was dissolved in toluene and the

solution was distilled again at 80 °C under vacuum to remove the solvent. This step was repeated 3 times.

Example 3. HA-PLA synthesis

Hyaluronic acid in CTA salt-form (HA-CTA) and poly(lactic acid) mono-acyl chloride (PLA-COCl) were reacted in a molar ratio of 2:1 of PLA-COCl in relation to HA-CTA.

2.03 g of PLA-COCl dissolved in 50 ml DMSO was added dropwise to a solution of 0.512 g of HA-CTA in 70 ml DMSO. After addition, the solution was mixed at room temperature overnight. Using a rotavaporator to remove DMSO the solution was concentrated until a solid product was obtained. The product was washed successively with ethanol and acetone. The final product was insoluble in water but swelled in DMSO.

The IR spectrum of the final product (KBr disk) is shown in figure 5. It looks like the spectrum of HA-H (shown in figure 4) except for the peak at 1735 cm⁻¹ which correspond to a C=O bond. However, the intensity of this peak is larger and is assignable to poly(lactic acid). The IR spectrum of PLA is shown in figure 3.

¹³C NMR seems to be the more efficient method for the characterisation of the final product, since all peaks in the ¹³C NMR spectrum of HA are clearly identified as follows; the carbon atoms of the D-glucuronic acid are labelled "U", and those of N-acetyl-D-glucosamine acid are labelled "N":

U6 → 174 ppm	N7 → 175 ppm
U1 → 100 or 104 ppm	N1 → 100 or 104 ppm
	N8 → 22 ppm
U2,3,4,5 → 54 at 83 ppm	N3,4,5 → 54 at 83 ppm
	N2 → 54,5 ppm
	N6 → 60,5 ppm

The ¹³C NMR spectrum of the final product (figure 6) shows all the peaks of HA, PLA (16, 57 and 170 ppm), and cetylammonium (CTA) counter-ions (13, 29, 52 ppm).

The evaporation of DMSO to solidify the product may gradually bring the PLA and HA closer together in space, which may then lead to a better coupling reaction. Differences in the chain lengths of HA and PLA may influence the substitution ratio in the reaction.

Example 4. HA-PLA-HA synthesis

PLA di-acyl chloride in DMSO was mixed with HA (TBA or CTA form) at room temperature during 1 night, as described above. The solution was then concentrated and the product was purified by precipitation in ethanol, and finally washed with acetone. The final product is insoluble in water.

The products removed by these two solvents were analysed by IR spectra, as shown in figures 8 and 9. The two spectra display peaks that are characteristic of both PLA and HA. We assume the washes eliminated some PLA linked to HA.

The IR spectra of the final products from HA-CTA and HA-TBA (figures 10 and 11) also look like the spectrum of HA-H except for the peak at 1736 cm⁻¹, which corresponds to the C=O bond of PLA.

Example 5. HA-CTA synthesis

A warm solution (40°C) of cetyltrimethylammonium bromide is added dropwise in a warm solution (40°C) of 0,155 g of HA-Na. The white precipitate is filtered, washed with warm water to remove NaBr and excess of cetylammmonium bromide and lyophilised.

The IR spectrum (figure 12) of the final product, which is soluble in DMSO, shows presence of CTA.

Example 6. HA-PLA synthesis in a 1:1 molar ratio

Typically, 0,64g of activated lactic acid oligomers was mixed with 1g of HA-CTA in DMSO overnight at room temperature. DMSO was removed and the precipitate was washed two times with ether, three times with ethanol, and two times with acetone. The recovered products were finally dried under vacuum overnight. ¹H NMR of the final product (figure 13) shows the presence of remnant CTA.

Example 7. HA-PLA dialysis

HA-PLA with remnant CTA were dissolved in a phosphate buffer solution (pH=7,4 and concentration = 0,5M) mixed with DMSO in a 2/1 volume ratio. This solution was dialysed (cut-off = 6000-8000) successively against water, DMSO, ethanol and water. After this treatment, the solution was freeze-dried, and the final compound was analysed by NMR (figure 14).

Example 8. HA-PLA blank test

A blank test was made by reacting HA and PLA without any prior activation of the oligomers with SOCl₂. A solution containing 271 mg OLA in 20 ml DMSO was added dropwise to a solution of 211 mg of HA in 40 ml of DMSO. This solution was stirred for 3h at room temperature and the DMSO was removed by evaporation. A light yellow solid was obtained. This solid was dissolved in DMSO overnight. A precipitate appeared. This insoluble part was separated from the solution and washed with acetone. The white solid obtained was dried and analysed by NMR.

Acetone was slowly added to the remaining solution to yield a novel precipitate. This precipitate was collected, dried and also analysed by NMR. No peaks of PLA were visible on the NMR spectrum.

This confirms that PLA is chemically linked to HA when activation of PLA by thionyl
5 chloride is used.

CLAIMS

1. A product comprising hyaluronic acid or a salt thereof, wherein the hyaluronic acid or salt thereof is partially or fully linked or crosslinked with a polymer of an alpha hydroxy acid, preferably of poly(lactic acid).

2. The product according to claim 1, wherein the hyaluronic acid or salt thereof is recombinantly produced, preferably by a Gram-positive bacterium, more preferably by a bacterium of the genus *Bacillus*.

3. The product according to claim 1 or 2, wherein the hyaluronic acid or salt thereof has a molecular weight in the range of between 300,000 and 3,000,000; preferably in the range of between 400,000 and 2,500,000; more preferably in the range of between 500,000 and 2,000,000; and most preferably in the range of between 600,000 and 1,800,000.

4. The product according to any of claims 1 - 3, which comprises an inorganic salt of hyaluronic acid, preferably sodium hyaluronate, potassium hyaluronate, ammonium hyaluronate, calcium hyaluronate, magnesium hyaluronate, zinc hyaluronate, or cobalt hyaluronate.

5. The product according to any of claims 1 - 4, wherein the linked or crosslinked hyaluronic acid or salt thereof comprises esters of a polymeric alpha hydroxy acid, preferably of poly(lactic acid).

6. The product according to any of claims 1 - 5, which is dried and comprises less than 5% moisture, preferably less than 2%, and most preferably less than 1% moisture, as determined herein.

7. The product according to any of claims 1 - 6, which also comprises an active ingredient.

8. The product according to claim 7, wherein the active ingredient is a pharmacologically active substance.

9. The product according to any of claims 1 - 8, which also comprises a water-soluble excipient, preferably lactose.

10. A composition comprising a product as defined in any of claims 1 – 9 and an active ingredient, preferably the active ingredient is a pharmacologically active agent.

11. The composition according to claim 10, which also comprises a water-soluble excipient, preferably lactose.

12. A pharmaceutical composition comprising an effective amount of a product as defined in any of claims 1 – 9, together with a pharmaceutically acceptable carrier, excipient or diluent.

13. A pharmaceutical composition comprising an effective amount of a product as defined in any of claims 1 – 9 as a vehicle, together with a pharmacologically active agent.

14. A cosmetic article comprising as an active ingredient an effective amount of a product as defined in any of claims 1 – 9.

15. A sanitary, medical or surgical article comprising a product as defined in any of claims 1 – 9; preferably the article is a surgical sponge, a wound healing sponge, or a part comprised in a band aid or other wound dressing material.

16. A medicament capsule or microcapsule comprising a product as defined in any of claims 1 – 9.

17. A method of producing a product comprising hyaluronic acid or a salt thereof, wherein the hyaluronic acid is partially or fully linked or crosslinked with a polymer of an alpha hydroxy acid, preferably poly(lactic acid), the method comprising the step of:

a) reacting hyaluronic acid or a salt thereof with a mono-acyl chloride or di-acyl chloride of the polymer of the alpha hydroxy acid in an organic solvent, preferably in DMSO.

18. The method according to claim 17, wherein the hyaluronic acid or salt thereof is recombinantly produced, preferably by a Gram-positive bacterium, more preferably by a bacterium of the genus *Bacillus*.

19. The method according to claim 17 or 18, wherein the hyaluronic acid or salt thereof has a molecular weight in the range of between 300,000 and 3,000,000; preferably in the range of between 400,000 and 2,500,000; more preferably in the range of between 500,000 and 2,000,000; and most preferably in the range of between 600,000 and 1,800,000.

20. The method according to any of claims 17 - 19, wherein the product comprises an inorganic salt of hyaluronic acid, preferably sodium hyaluronate, potassium hyaluronate, ammonium hyaluronate, calcium hyaluronate, magnesium hyaluronate, zinc hyaluronate, or cobalt hyaluronate.

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21. The method according to any of claims 17 – 20, wherein the linked or crosslinked hyaluronic acid or salt thereof comprises esters of a polymeric alpha hydroxy acid, preferably of poly(lactic acid).

10 22. In a method of performing procedures in ophtalmology, the improvement which comprises the use of a product as defined in any of claims 1 - 9, or a composition as defined in any of claims 10 – 13.

15 23. In a method of performing procedures in the treatment of osteoarthritis, the improvement which comprises the use of a product as defined in any of claims 1 - 9, or a composition as defined in any of claims 10 – 13.

20 24. In a method of performing procedures in the treatment of cancer, the improvement which comprises the use of a product as defined in any of claims 1 - 9, or a composition as defined in any of claims 10 – 13.

25 25. In a method of performing transdermal administration of a pharmacologically active agent, the improvement which comprises the use of a product as defined in any of claims 1 - 9, or a composition as defined in any of claims 10 – 13.

26. In a method of performing dermal administration of a pharmacologically active agent, the improvement which comprises the use of a product as defined in any of claims 1 - 9, or a composition as defined in any of claims 10 – 13.

30 27. In a method of performing dermal administration of a cosmetic, the improvement which comprises the use of a product as defined in any of claims 1 - 9, or a composition as defined in any of claims 10 – 13.

35 28. An ophtamological method of using a product as defined in any of claims 1 - 9, or a composition as defined in any of claims 10 – 13.

29. A method of treating osteoarthritis, comprising administering an effective amount of a product as defined in any of claims 1 - 9 or a composition as defined in any of claims 10 - 13 to a mammal, preferably the administering is dermal, transdermal, oral, or by injection.

5 30. A method of treating a wound, comprising administering an effective amount of a product as defined in any of claims 1 - 9 or a composition as defined in any of claims 10 - 13 to a mammal.

31. Use of a product as defined in any of claims 1 - 9 or a composition as defined in any
10 of claims 10 - 13 for the manufacture of a medicament for the treatment of osteoarthritis.

32. Use of a product as defined in any of claims 1 - 9 or a composition as defined in any of claims 10 - 13 for the manufacture of a medicament for an opthalmological treatment.

15 33. Use of a product as defined in any of claims 1 - 9 or a composition as defined in any of claims 10 - 13 for the manufacture of a medicament for the treatment of cancer.

34. Use of a product as defined in any of claims 1 - 9 or a composition as defined in any of claims 10 - 13 for the manufacture of a medicament for the treatment of a wound.

20

35. Use of a product as defined in any of claims 1 - 9 or a composition as defined in any of claims 10 - 13 for the manufacture of a medicament for angiogenesis.

36. Use of a product as defined in any of claims 1 - 9 or a composition as defined in any
25 of claims 10 - 13 for the manufacture of a moisturizer.

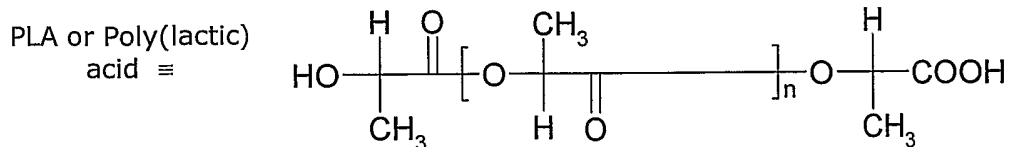
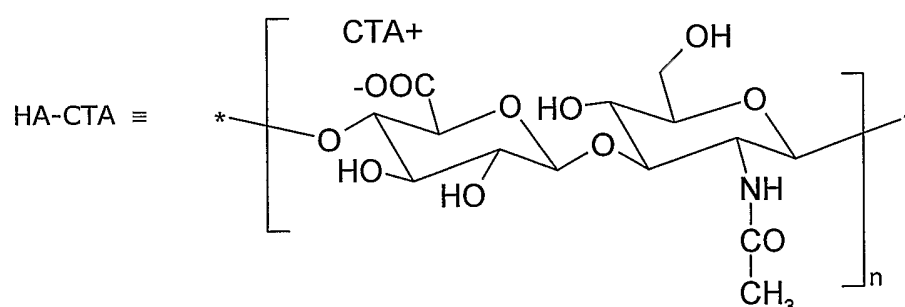
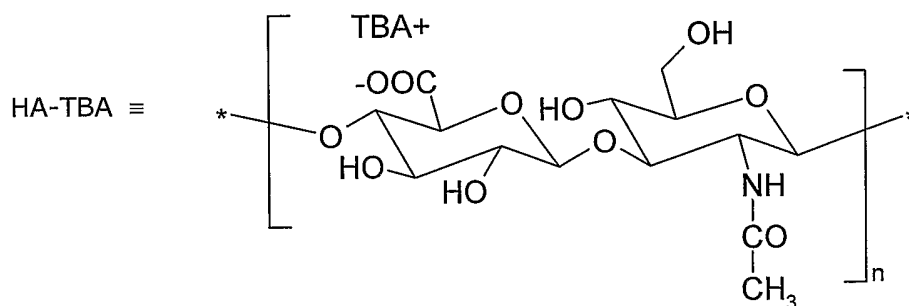
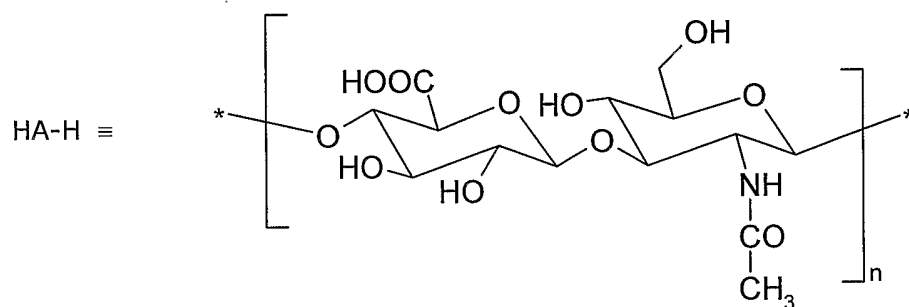
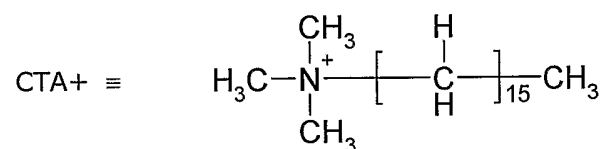
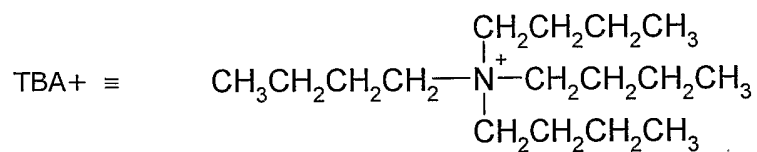
Figure 1/14

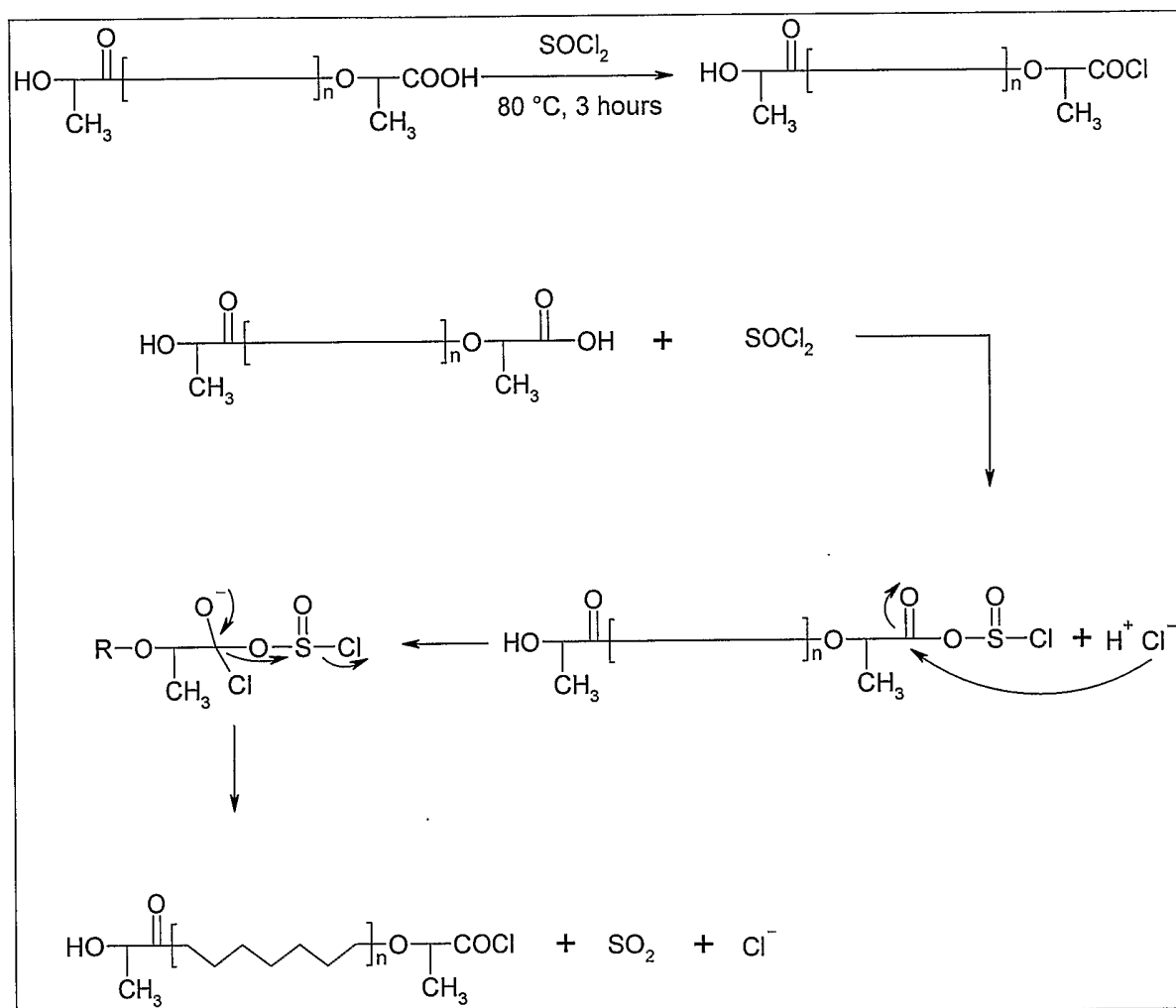
Figure 2/14

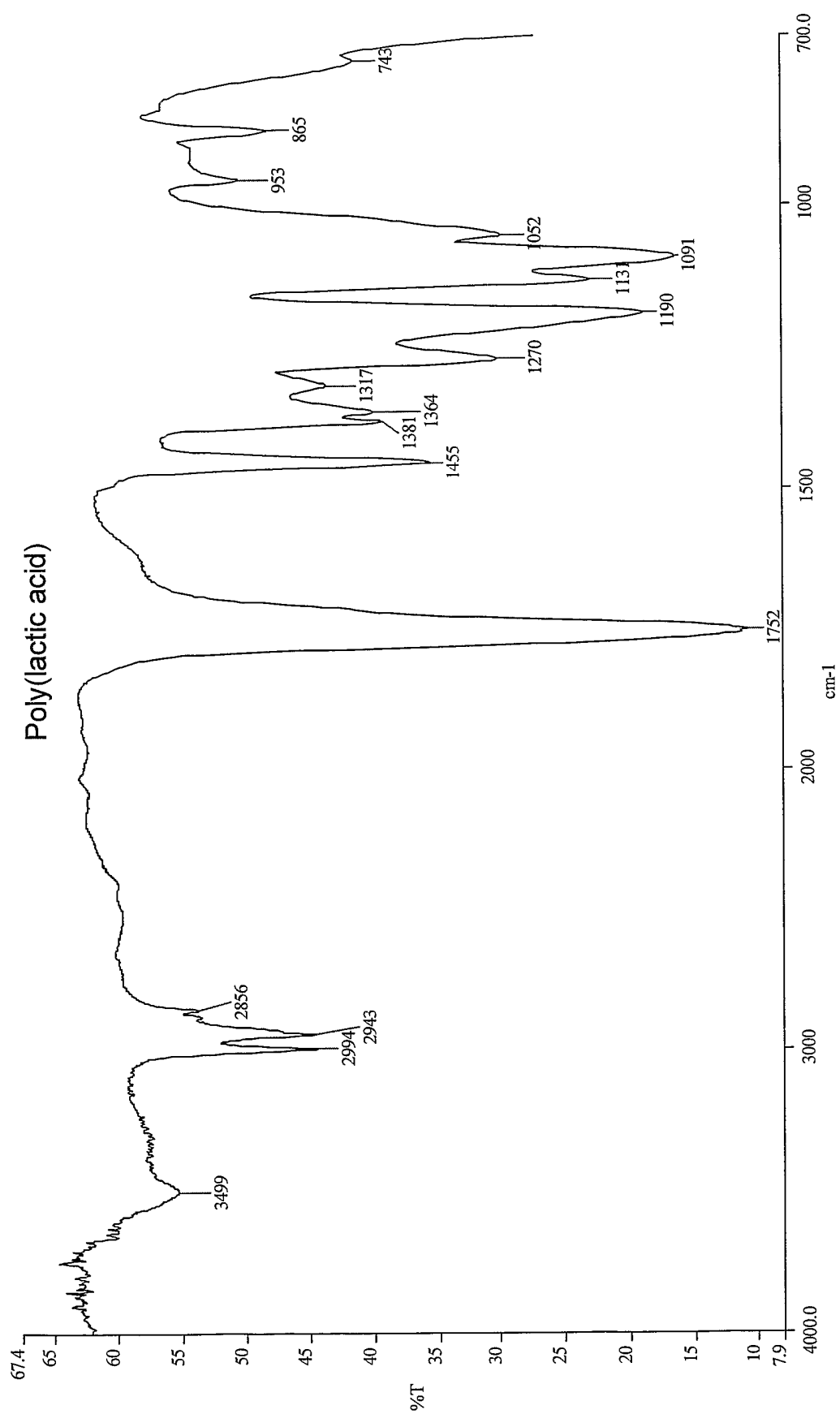
Figure 3/14

Figure 4/14

HA-H = Hyaluronic acid (proton form)

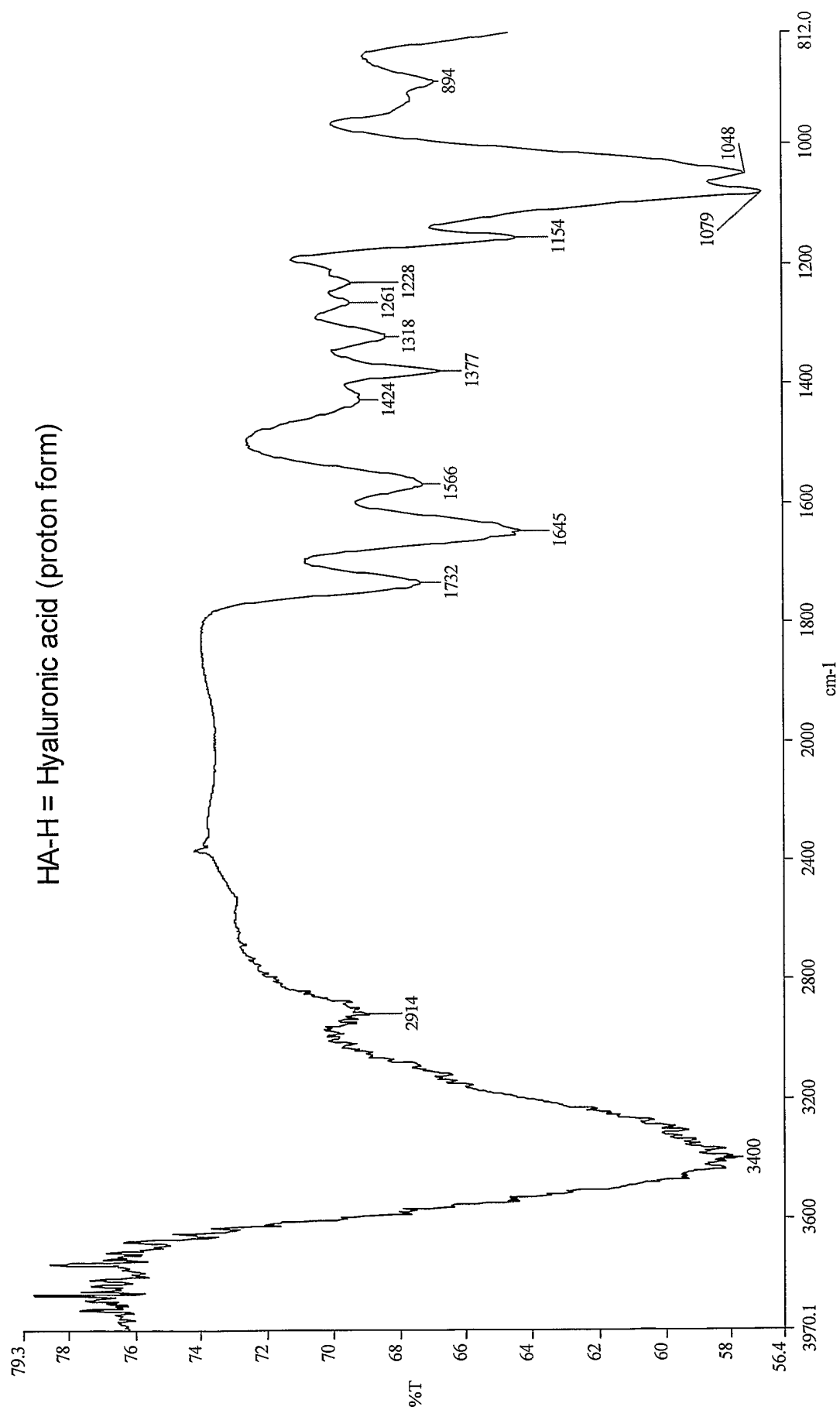


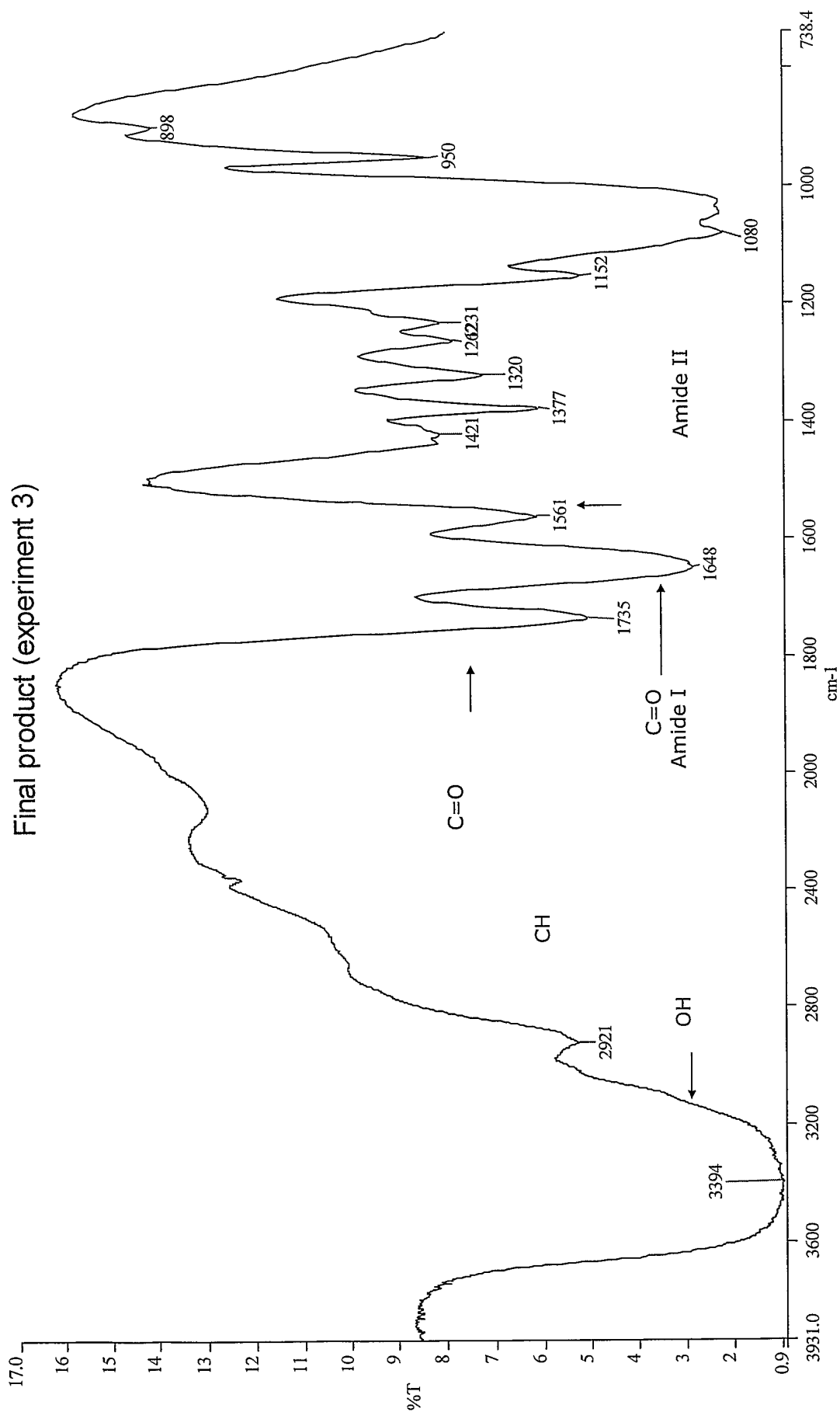
Figure 5/14

Figure 6/14

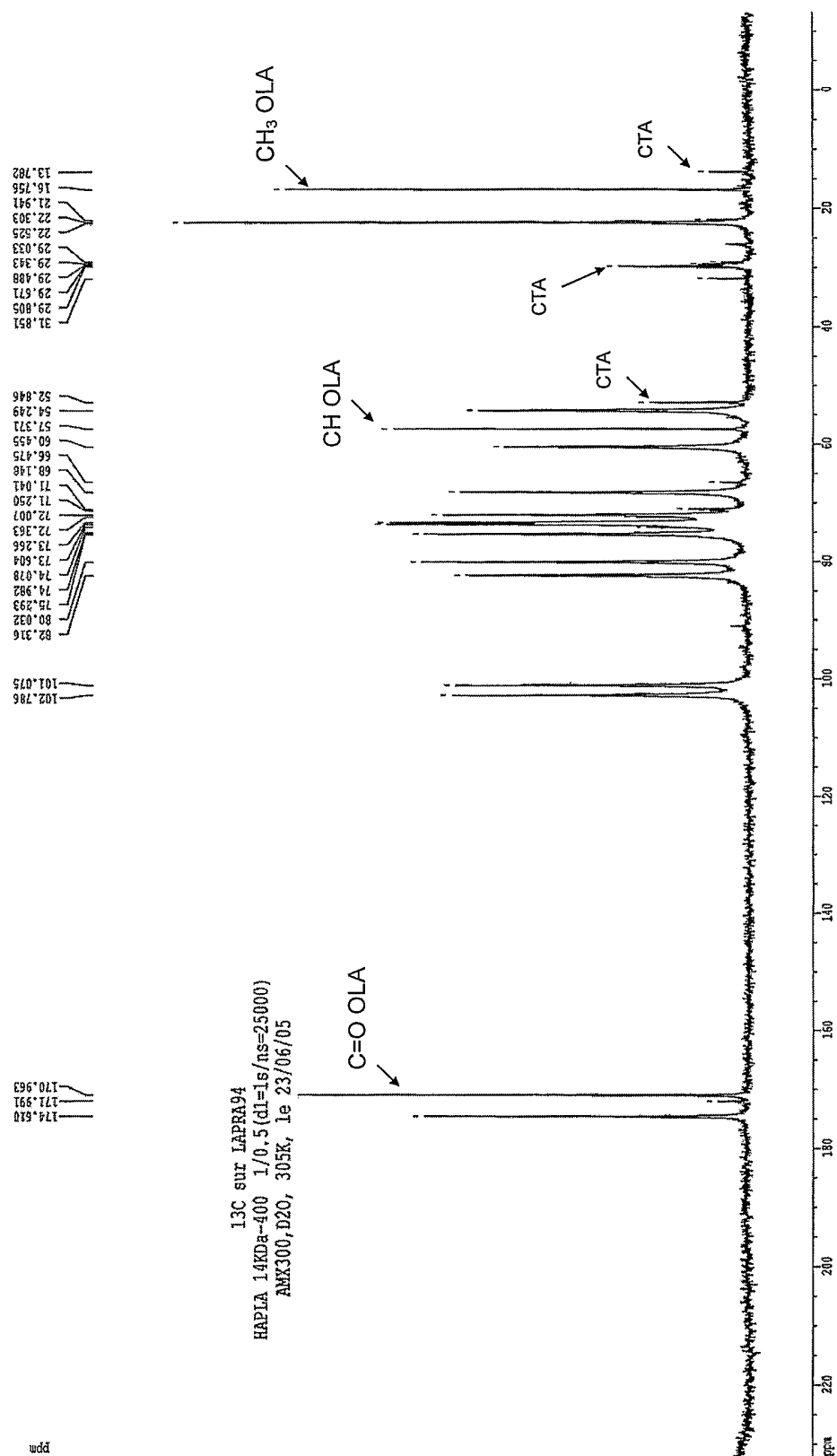


Figure 7/14

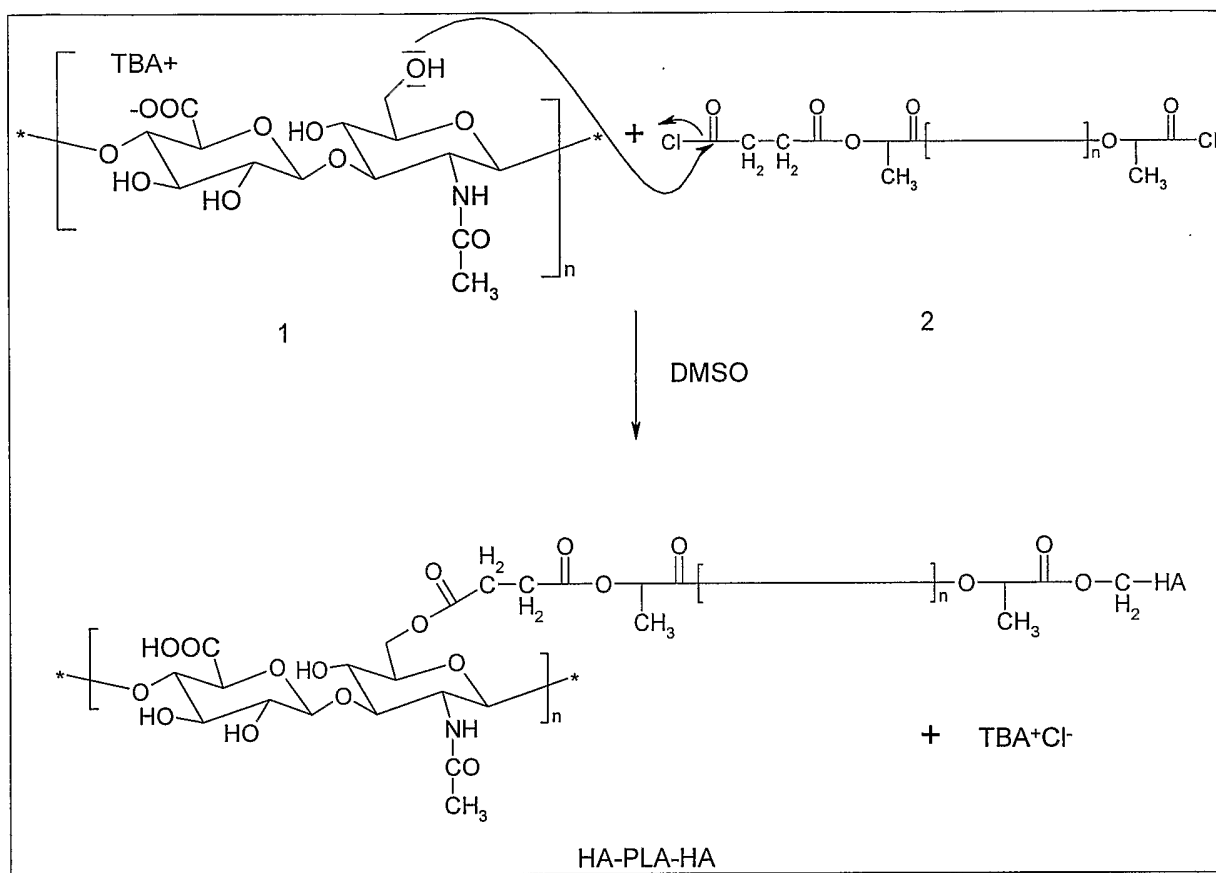


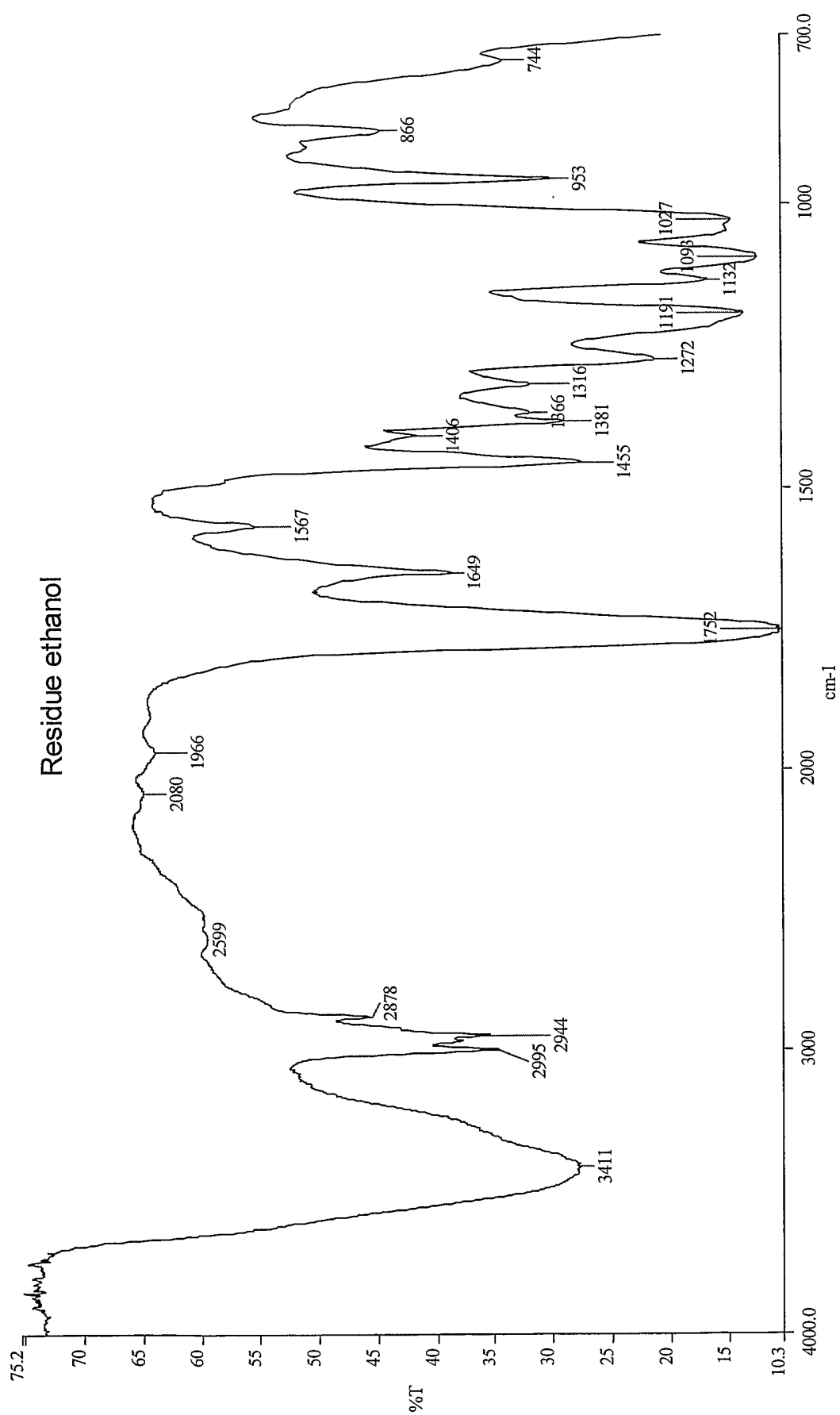
Figure 8/14

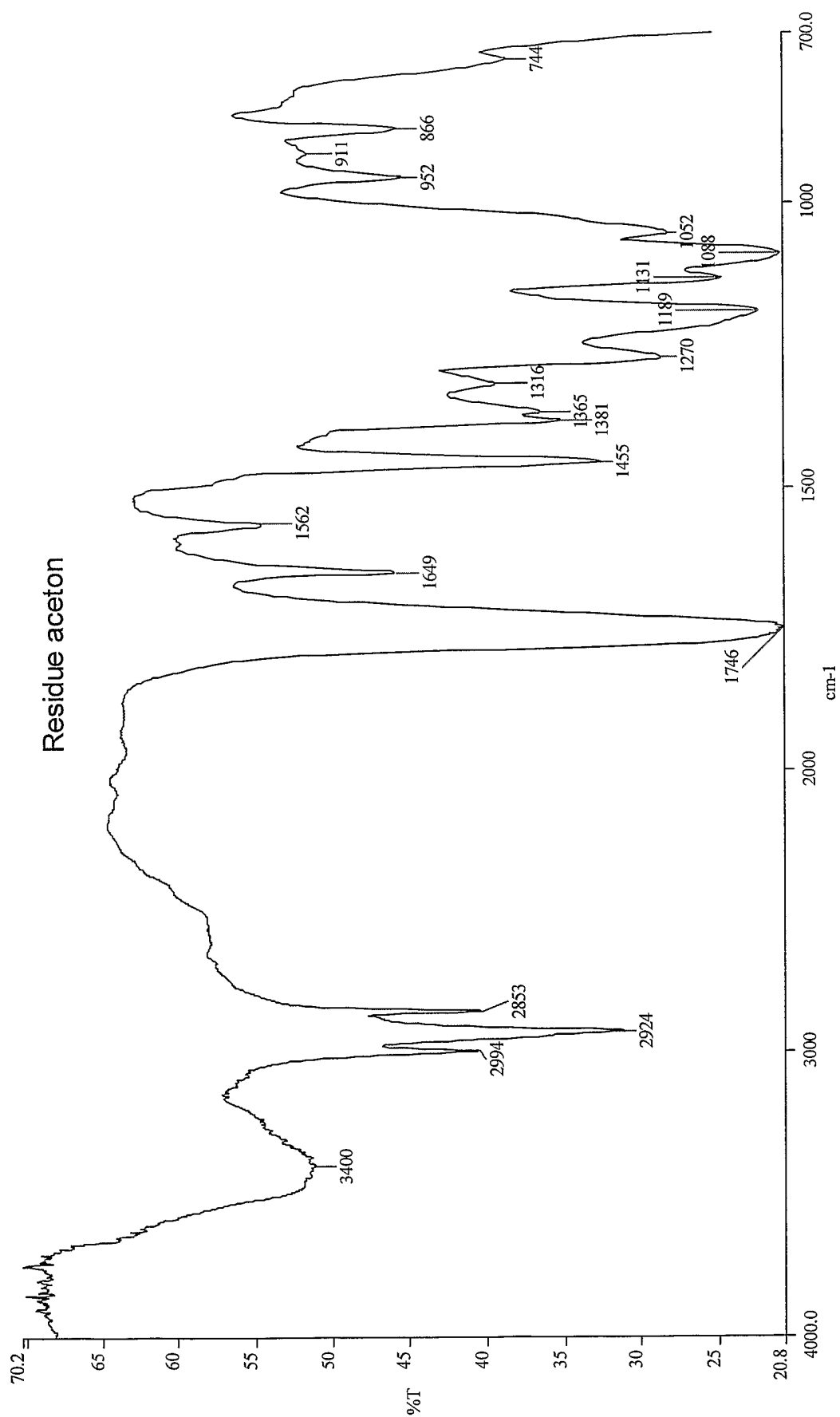
Figure 9/14

Figure 10/14

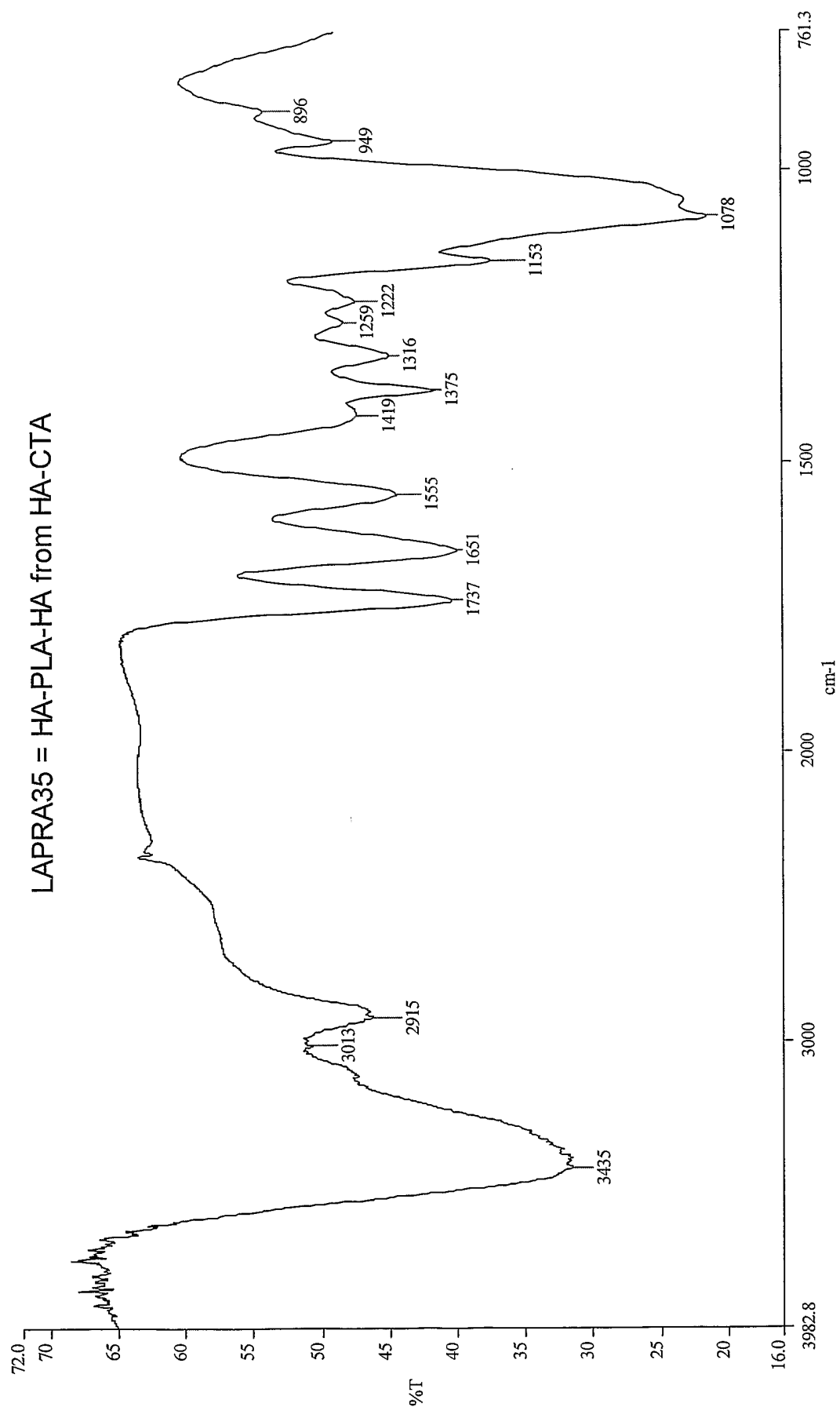


Figure 11/14

LAPRA36 = HA-PLA-HA from HA-TBA

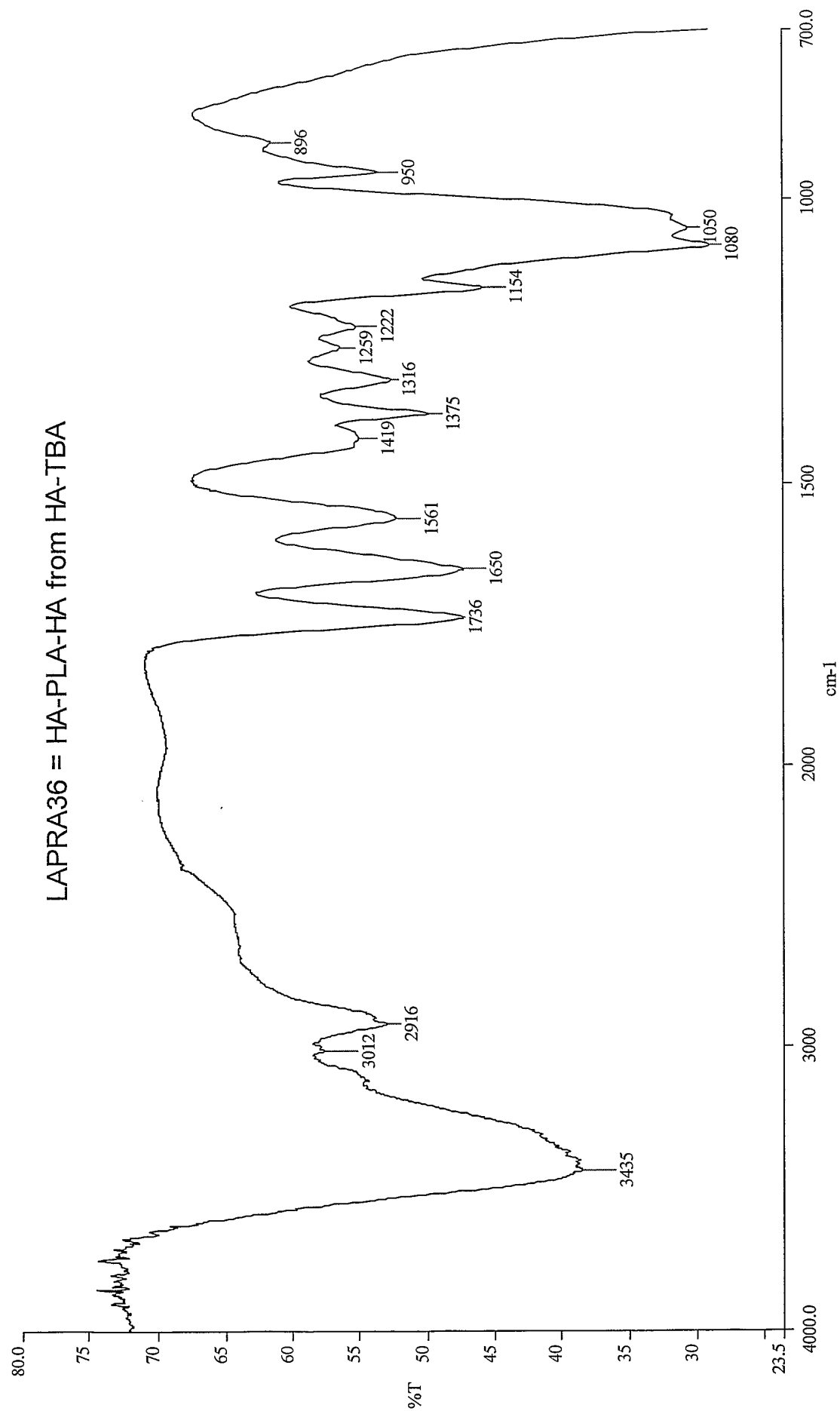
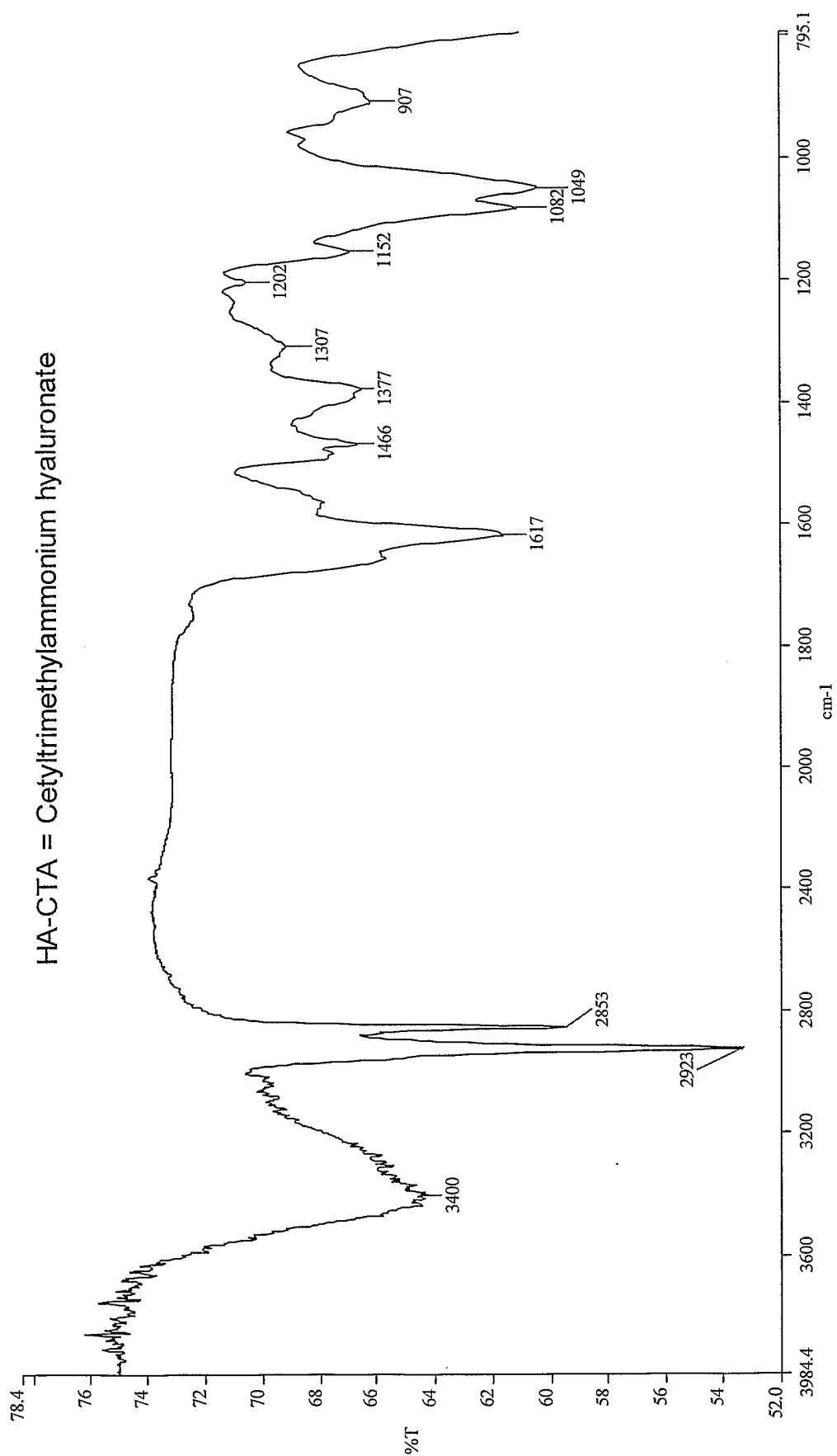


Figure 12/14

HA-CTA = Cetyltrimethylammonium hyaluronate



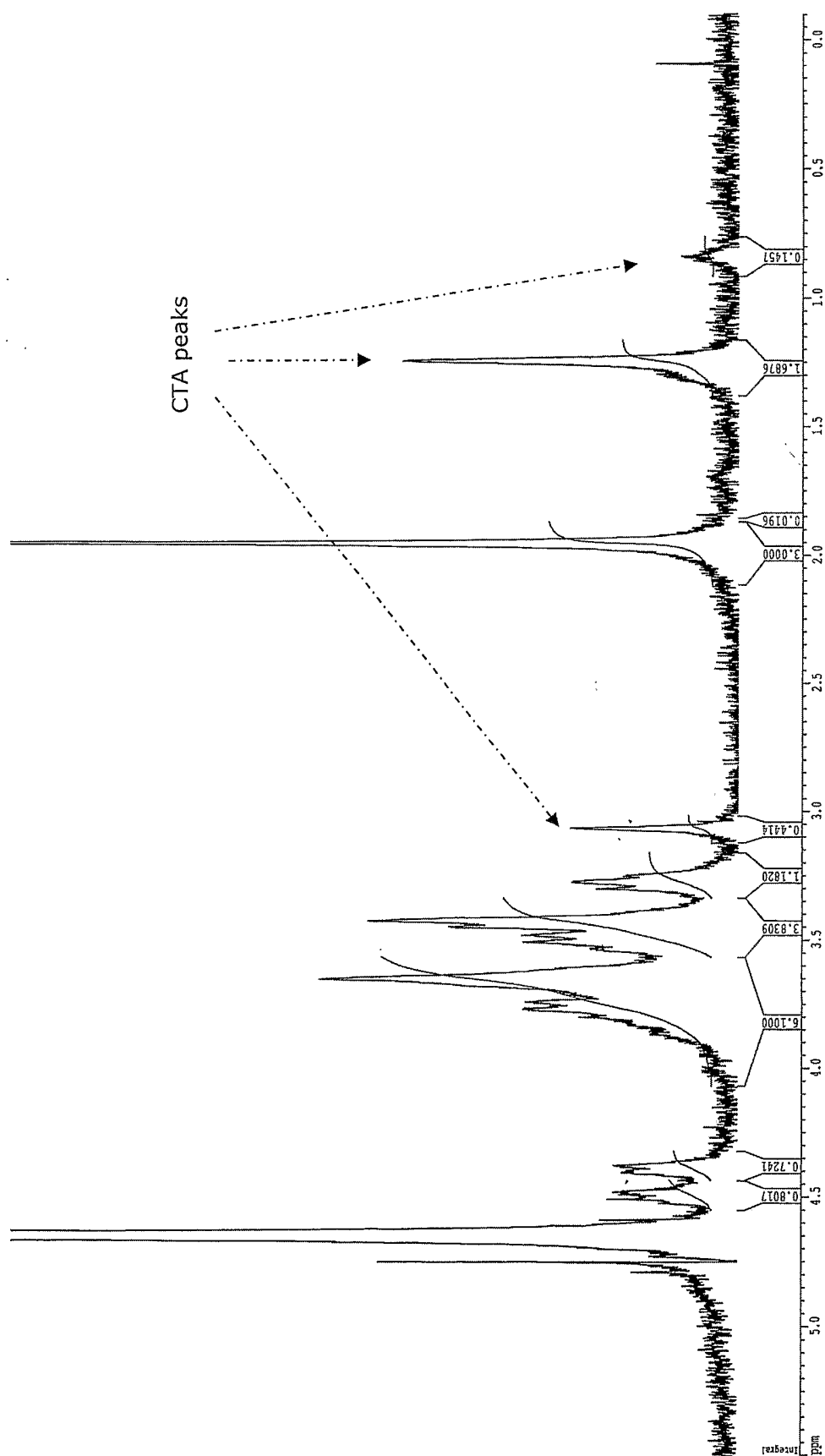
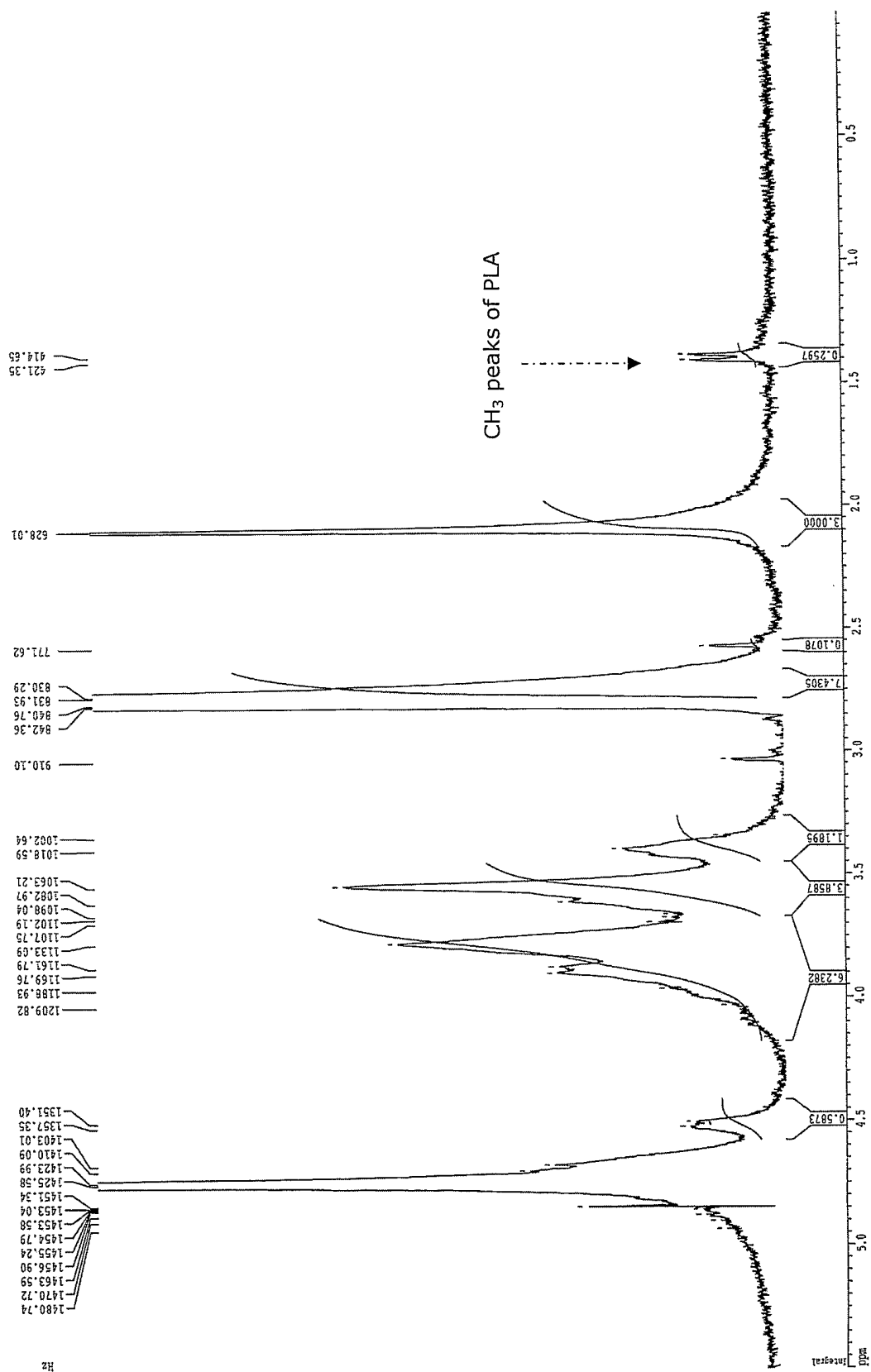


Figure 14/14



INTERNATIONAL SEARCH REPORT

International application No
.../DK2005/000826

A. CLASSIFICATION OF SUBJECT MATTER

INV. C08G81/00 C08B37/08 A61K31/728 A61K8/73 A61K47/36
A61K9/50 A61L15/28 A61L17/00 A61L31/06

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C08G C08B

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, PAJ, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 01/88019 A (CENTRE NATIONAL DE LA RECHERCHE SCIENTIFIQUE ; GREF, RUXANDRA; PONCHEL) 22 November 2001 (2001-11-22)	1,3-13, 16,17, 19-22, 24-26, 28,32-35
Y	page 6, line 1 - line 22 page 10, line 30 - line 31 page 17, line 1 - line 17 page 18, line 24 - line 27 page 20, line 1 - line 9 page 28; example 11 claims	2,18
Y	----- -/--	14,15, 23,27, 31,36



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents :

- *A* document defining the general state of the art which is not considered to be of particular relevance
- *E* earlier document but published on or after the international filing date
- *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the international filing date but later than the priority date claimed

- *T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- *X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- *Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- *&* document member of the same patent family

Date of the actual completion of the international search

28 April 2006

Date of mailing of the international search report

11/05/2006

Name and mailing address of the ISA/

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Fax: (+31-70) 340-3016

Authorized officer

Mazet, J-F

INTERNATIONAL SEARCH REPORT

International application No

.../DK2005/000826

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 03/054163 A (NOVOZYMES BIOTECH, INC; NOVOZYMES BIOPOLYMER A/S) 3 July 2003 (2003-07-03) abstract page 1, line 32 - page 2, line 12 page 7, line 5 - line 10 page 7, line 19 - line 23 -----	2,18
Y	WO 00/27887 A (AQUISITIO S.P.A; BARBUCCI, ROLANDO; RAPUOLI, ROBERTO) 18 May 2000 (2000-05-18) page 1, line 21 - page 2, line 25 page 3, line 14 - line 19 page 8, line 12 - page 10, line 4 -----	14,15, 23,27, 31,36
P,X	WO 2005/023906 A (CHUGAI SEIYAKU KABUSHIKI KAISHA; HIRAKURA, TAI; NAKAMURA, TERUO; SHIMO) 17 March 2005 (2005-03-17) the whole document -----	1-28, 31-36

INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK2005/000826

Box II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 29, 30
because they relate to subject matter not required to be searched by this Authority, namely:
Methods of treatment of human or animal body by therapy (Rule 39.1(iv);
Subject Matter Under Article 17(2)(a)(i))
2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

DK2005/000826

Patent document cited in search report		Publication date	Patent family member(s)	Publication date
WO 0188019	A	22-11-2001	AU 6242701 A CA 2408870 A1 EP 1285021 A1 FR 2809112 A1 JP 2004521152 T US 2004013626 A1	26-11-2001 22-11-2001 26-02-2003 23-11-2001 15-07-2004 22-01-2004
WO 03054163	A	03-07-2003	AU 2002366711 A1 CA 2471148 A1 CN 1636052 A CZ 20040765 A3 EP 1572895 A2 JP 2005525091 T	09-07-2003 03-07-2003 06-07-2005 15-12-2004 14-09-2005 25-08-2005
WO 0027887	A	18-05-2000	AT 225371 T AU 757102 B2 AU 2660000 A BR 9915235 A CA 2350667 A1 CN 1131241 C CZ 20011650 A3 DE 69903351 D1 DE 69903351 T2 DK 1144459 T3 EP 1144459 A2 ES 2184521 T3 HK 1041011 A1 HU 0104296 A2 IT MI982440 A1 JP 2002529550 T NO 20012315 A NZ 511306 A PT 1144459 T RU 2230752 C2 TR 200101324 T2 US 6831172 B1 ZA 200103799 A	15-10-2002 30-01-2003 29-05-2000 24-07-2001 18-05-2000 17-12-2003 12-09-2001 07-11-2002 03-07-2003 03-02-2003 17-10-2001 01-04-2003 08-10-2004 28-03-2002 11-05-2000 10-09-2002 06-07-2001 30-05-2003 31-01-2003 20-06-2004 21-09-2001 14-12-2004 12-08-2002
WO 2005023906	A	17-03-2005	NONE	