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(54) **Title**: LIPOCHITOOLIGOSACCHARIDES STIMULATING ARBUSCULAR MYCORRHIZAL SYMBIOSIS

(57) **Abstract**: The invention relates to lipochitooligosaccharides obtainable from arbuscular mycorrhizal fungi, and which are useful for stimulating arbuscular mycorrhizal symbiosis, and lateral root formation.

LIPOCHITOOLIGOSACCHARIDES STIMULATING ARBUSCULAR MYCORRHIZAL SYMBIOSIS

The invention relates to lipochitooligosaccharides involved in arbuscular mycorrhizal symbiosis, and to their applications.

Arbuscular mycorrhizal (AM) fungi have established symbiotic associations with plant roots for over 400 million years, since the appearance of the earliest land plants, suggesting that AM fungi assisted plants in their colonization of land (Remy et al., 1994). This group of fungi, recently renamed Glomeromycota, is one of the most widely distributed and AM associations are widely distributed throughout the plant kingdom including angiosperms, gymnosperms, pteridophytes and some bryophytes (Smith and Read, 2008). Among the angiosperms, at least 80% of the species can form AM symbioses, the only major exceptions being Crucifers and Chenopodiaceae. AM fungi are able to transfer rare or poorly soluble mineral nutrients such as phosphorus, zinc and copper from the soil to the plant, which in turn provides carbohydrates to the fungus. This exchange of nutrients can be of critical importance when soil fertility and water availability are low, conditions that severely limit agricultural production in most parts of the world (Smith and Read, 2008).

Another known symbiotic association between plants and soil microorganisms is the rhizobial symbiosis. In contrast with the arbuscular mycorrhizal symbiosis, which is broadly distributed among plants, the rhizobial symbiosis is restricted to legumes, and instead of fungi, it involves nitrogen-fixing bacteria collectively called rhizobia, which belong to several genera including *Rhizobium, Bradyrhizobium, Azorhizobium,* and *Sinorhizobium.* The rhizobial symbiosis results in the formation of specific structures, the nodules, on the roots of the legume host. The nodules provide an appropriate environment for rhizobia, allowing them to fix molecular nitrogen and to provide combined nitrogen to the legume host. The initiation of the Rhizobium-legume association depends on symbiotic signals that are produced by both symbiotic partners. The signals released by the plant are usually flavonoids excreted in root exudates. These flavonoids interact with rhizobial transcription factors of the NodD family, which activate the transcription of nodulation (*nod*) genes involved in the production of the bacterial signaling molecules termed Nod factors (Dénarié et al., 1996). Nod factors share a common basic structure consisting of a chitin backbone of four or five beta-1,4-linked N-acetylglucosamine residues, N-acylated at the nonreducing end with a fatty acid group of variable length and degree of unsaturation. This basic structure can be further N-methylated at the nonreducing end, and can also be O-substituted at the nonreducing end and/or at the reducing end. This variety of substituents provides a broad diversity of Nod factors with different structures (for the description of diverse structures of Nod factors see Dénarié et al., 1996; D'Haeze et al., 2002). The specificity within the legume/rhizobial interaction (i.e. a given species of rhizobia forms nodules on certain species of legumes) is the result of this diversity.

Genetic dissection of the pathway involved in Nod factor signaling in roots of the model legume *Medicago truncatula* has identified a number of genes involved in this pathway (Stacey et al., 2006). There is growing evidence that the Nod factor receptors are receptor-like kinases with extracellular sugar-binding LysM domains, such as those encoded by the *NFP* and *LYK3* genes of *Medicago truncatula.* The binding of a Nod factor to its receptor induces a downstream signaling cascade, including the rapid influx of calcium ions and calcium spiking, and expression of specific nodulin genes. These downstream events involve in particular genes encoding proteins involved in calcium signaling, such as *DMI1*, *DMI2* and *DMI3* of *Medicago truncatula* which encode respectively a cation channel, a leucine rich-repeat receptor-like kinase, and a Ca^{2+}/calmodulin-dependent protein kinase, and genes encoding proteins involved in the control of gene expression, such as *NSP1* and *NSP2* which encode transcription factors.

Although AM fungi are both agriculturally and ecologically extremely important, the cellular and molecular mechanisms which control the formation of the mycorrhizal symbiosis, are far less known than those involved in rhizobial symbiosis.

It has been shown in *M. truncatula* that the nodulation and mycorrhizal programs share at least three components (Catoira et al. 2000), namely the products of the *DMI1*, *DMI2* and *DMI3* genes involved in calcium signaling.

However, the events taking place upstream and downstream this calcium signaling are still poorly characterized in the case of the arbuscular mycorrhizal symbiosis, in particular those involved in early signaling and leading to the recognition between the plant and the fungal partners. The study of these events has been hampered by the facts that the fungal partner is an obligatory symbiont which cannot be grown in pure culture in the absence of living plants, and by the absence of genetic tools available for this group of fungi (Harrison, 2005). However it has been shown recently that diffusible signals are exchanged between the symbionts prior to physical interaction. On the plant side, compounds of the apocarotenoid family, strigolactones, can be secreted in root exudates and stimulate ramifications in hyphae from AM fungi germinating spores, signaling a physiological switch to active presymbiotic fungal growth (Akiyama et al., 2005; Besserer et al., 2006). On the fungal side, the existence of diffusible compounds produced by AM fungi and able to activate plant responses associated to endomycorrhization program, has also been reported (Kosuta et al., 2003; Weidmann et al., 2004; Navazio et al., 2007). More specifically, a series of experiments performed with *M. truncatula* have recently shown that AM fungi produce diffusible compounds that are able to stimulate the expression of diverse plant responses. Three species of *Gigaspora* and one species of *Glomus* could trigger through a cellophane membrane the induction of expression of the *MtENOD11* symbiotic gene in seedling roots (Kosuta et al., 2003). Three fungal pathogens did not elicit the same response, supporting the hypothesis that the response was induced by a specific AM fungal signal molecule. Similarly,

an AM fungus, *Glomus intraradices*, was shown to activate through a membrane the transcription of plant genes whose expression depends on the *DMI3* symbiotic gene (Weidmann et al. 2004). In addition, a diffusible signal from AM fungi was found to elicit a transient cytosolic calcium elevation in soybean cell cultures and the up-regulation of genes related to *DMI1*, *DMI2* and *DMI3* (Navazio et al., 2007).

Olah et al. (2005) reported that Nod factors from *Sinorhizobium meliloti*, the rhizobial symbiont of *M. truncatula*, were able to stimulate mycorrhization and lateral root formation in *M. truncatula.* The stimulation of lateral root formation was also observed with diffusible factors from arbuscular mycorrhizal fungi (Myc factors), but not with Nod factors from rhizobial species (*Sinorhizobium fredii* and *Rhizobium leguminosarum*), which cannot nodulate *Medicago* sp. They also reported that all the genes of the Nod factor signaling pathway presently identified, including in particular the *NFP* gene encoding the putative Nod factor receptor, as well as the *DMI3* and *NSP1* genes are required for stimulation of lateral root formation by Nod factors, but not by Myc factors, which require only the *DMI1* and *DMI2* genes. On the basis of these observations, these authors proposed a model explaining the stimulation of mycorrhization and of lateral root formation in legumes by both Myc factors and Nod factors. According to this model, Myc factors and Nod factors, which are recognized by different cell surface receptors, activate a common DMI1/DMI2/DMI3 signalling pathway. In the case of Myc factors, DMI1 and DMI2 are sufficient for stimulation of lateral root formation, while DMI3 is required for stimulation of mycorrhization.

Olah et al. also discussed the possible chemical nature of the Myc factors. They hypothesized that they are unlikely to be auxin-like compounds, since their effect on root development is different from the one observed with these compounds. They also suggested that their structure should be different from the structure of Nod factors, since they appeared to be discriminated by the NFP receptor.

Therefore, it appears that although the existence of diffusible "Myc factors" produced by AM fungi, and able to activate plant responses, is recognized in the art, the chemical nature of these factors has not been identified until now.

The inventors have now succeeded in purifying Myc factors from exudates from both mycorrhized roots and germinating spores of the AM fungus *Glomus intradices*. They have further determined their chemical structure, and shown that they efficiently stimulate the root system development.

The Myc factors purified by the inventors are a mixture of sulfated and non-sulfated lipochitooligosaccharides (LCOs); they share with the Nod factors a common basic chitin backbone of beta-1,4-linked N-acetylglucosamine residues, N-acylated at the nonreducing end with a fatty acid group. However the Myc factors have simpler structures than the Nod factors. The only O-substitution which is observed in Myc factors is O-sulfation at the reducing end of the molecule. No other O-substitutions such as O-acetyl, O-carbamoyl

at the non-reducing end, or O-fucosyl at the reducing end could be detected. The single N-substitution on the non-reducing terminal GlcNAc residue for Myc factors is the acylation by common fatty acids, mainly oleic (C18:1) and palmitic (C16:0) acids. In contrast the N-substitution of Nod factors is more complex. It is frequently a double substitution by a N-methyl group and a N-acyl group, as in rhizobial strains that nodulate most tropical legumes and legumes of the *Mimosoïdeae* sub-family. N-methylation is specified by the widespread *nodS* rhizobial gene (Dénarié et al., 1996). Alternatively, N-acylation by a specific poly-unsaturated fatty acid is the rule among rhizobia that nodulate temperate legumes of the Galegoid clade (Dénarié et al., 1996). In fact, LCOs having a structure as simple as the Myc factors that we have characterized, were not observed among the Nod factors synthesized by the various rhizobial strains studied so far (Dénarié et al., 1996; D'Haeze et al., 2002).

The invention thus provides a process for obtaining lipochitooligosaccharides from a fungus from the group Glomeromycota, wherein said process comprises obtaining exudates from plant roots mycorrhized with said fungus, or from germinating spores of said fungus, extracting said exudates with butanol, and recovering the butanol extract containing said lipochitooligosaccharides.

According to a preferred embodiment of the invention, said process comprises the further steps of subjecting said butanol extract to solid reverse-phase extraction on a C18 phase, with successive washes at 20%, 50% and 100% acetonitrile and recovering the fraction eluted at 50% acetonitrile containing said lipochitooligosaccharides.

Still more preferably, said process comprises the further steps of subjecting said fraction eluted at 50% acetonitrile to reverse-phase high-performance liquid chromatography on a C18 column, using a linear gradient of 20% to 100% acetonitrile, and recovering the fraction eluted at 30-48% of acetonitrile which contains sulfated lipochitooligosaccharides, and/or the fraction eluted at 64-72% of acetonitrile which contains non-sulfated lipochitooligosaccharides.

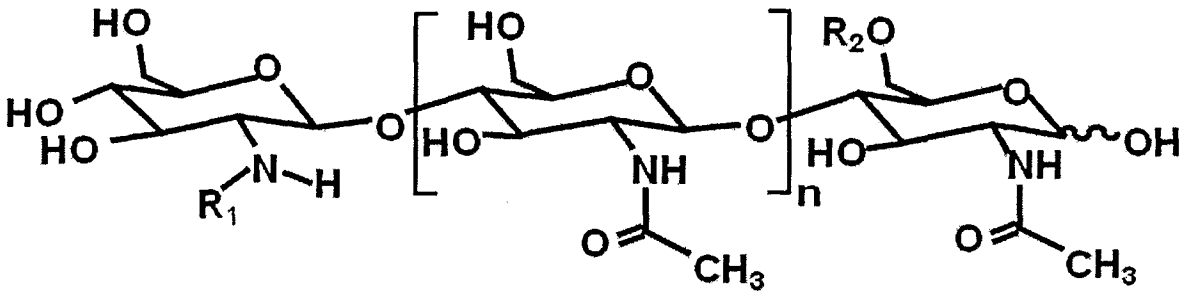
According to a particular embodiment of the invention, said fungus from the group Glomeromycota is *Glomus intraradices*.

Lipochitooligosaccharides of the invention can however also be extracted from other species of Glomeromycota producing them, using the extraction steps disclosed above, or variants thereof. The fungal extracts containing the lipochitooligosaccharides of the invention can easily be identified on the basis of their biological properties, using appropriate bioassays. In particular, one can use bioassays based on the properties of the lipochitooligosaccharides of the invention to stimulate the root system development: for instance, fungal fractions containing lipochitooligosaccharides of the invention, sulfated or not, can be identified on the basis of their ability to stimulate lateral root formation in *M. truncatula*. Bioassays for differentiating fungal fractions containing non-sulfated lipochitooligosaccharides from those containing sulfated lipochitooligosaccharides of the

invention are also available: for instance, fractions containing sulfated lipochitooligosaccharides of the invention are able to induce the expression of the *MtENOD11* gene in growing roots of *M. truncatula* while fractions containing non-sulfated lipochitooligosaccharides of the invention are able to induce root hair branching in vetch.

The invention also encompasses the preparations of lipochitooligosaccharides obtainable by the process of the invention.

According to a preferred embodiment, a preparation of lipochitooligosaccharides of the invention comprises a lipochitooligosaccharide defined by the formula (I) below:



(I)

wherein n = 0, 1, 2, 3, 4, or 5, preferably 2 or 3, R_1 represents a fatty acid chain containing 14 to 20 carbon atoms, which can be saturated, or mono-, di-, tri- tetra-, penta-, or hexaunsaturated, and R_2 represents H or SO_3H.

Advantageously, R_1 represents a chain of a fatty acid synthesized by arbuscular mycorrhizal fungi, in particular a C16 or C18 fatty acid chain, saturated, or mono- or di-unsaturated. Preferably, when said fatty acid chain is unsaturated, it comprises at least one cis-unsaturation. By way of non-limitative examples of preferred fatty acid chains, one can mention C16:0, C18:0, C16:1ω5, C16:1ω7, C18:1ω5, C18:1ω7, C18:1ω9, 18:2ω6,9, C20:0 iso, C20:1ω9 and C20:4ω6,9,12,15 .

A preparation of lipochitooligosaccharides of the invention may comprise a mixture of different lipochitooligosaccharides of formula (I) wherein R_1 represents different fatty acid chains. It may also comprise either lipochitooligosaccharides of formula (I) wherein R_2 represents H, or lipochitooligosaccharides of formula (I) wherein R_2 represents SO_3H, or, preferably a mixture of these both kinds of lipochitooligosaccharides.

Preparations of lipochitooligosaccharides of the invention can be obtained from arbuscular mycorrhizal fungi, as described above. The lipochitooligosaccharides can also be obtained by chemical synthesis and/or produced in genetically engineered bacterial cells. For instance, a chitooligosaccharide backbone, sulfated or not, can be synthesised in recombinant bacteria, as disclosed for instance by Samain et al. (1997, 1999) for the synthesis of Nod factor precursors, and subsequently acylated on the free amine group of the non-reducing terminal sugar. One can also use a mutant strain of a Rhizobiaceae bacterium,

genetically modified in order to express, among the structural genes of the Nod biosynthetic pathway, only those involved in the synthesis of the chitooligosaccharide backbone and those involved in the N-acylation of the non-reducing terminal glucosamine by an appropriate C16 or C18 fatty acid, and optionally those involved in the O-sulfation of the reducing terminal glucosamine.

The lipochitooligosaccharides of the invention can be used to stimulate mycorrhization, and thus have a broad range of applications in agriculture, horticulture and forestry, for most cultivated plants which can establish mycorrhization, and therefore possess Myc factor receptors. They can be used as well for plants grown under growth chamber, than in greenhouse or in field conditions. They can be used for instance for treating seeds, or added to inoculants containing arbuscular mycorrhizal fungi.

The lipochitooligosaccharides of the invention can also be used as stimulating additive to the culture media which are used for the production of mycorrhizal inoculants (i.e AM fungal spores or hyphae, or fragments of mycorrhized roots) by plants grown on soil or on hydroponic or aeropic conditions, or by co-culture of mycorrhizal fungi with excised roots.

In addition to their use for stimulating the arbuscular mycorrhizal symbiosis, the lipochitooligosaccharides of the invention can also be used to stimulate the root system development which is beneficial to improve water and mineral nutrition.

The lipochitooligosaccharides of the invention can be incorporated in compositions, for instance for coating seeds or an aqueous solution or suspension for treating the plant or the soil.

The invention also encompasses compositions containing lipochitooligosaccharides of the invention. Such compositions include in particular:

- a composition comprising seeds of a plant able to establish mycorrhization coated with a preparation of lipochitooligosaccharides of the invention;
- a composition comprising an inoculant of an arbuscular mycorrhizal fungus, and a preparation of lipochitooligosaccharides of the invention.

The lipochitooligosaccharides of the invention can optionally be combined with other active constituents, such as flavonoids, apocarotenoids such as strigolactones, or jasmonate which are plant compounds which have been reported to act as symbiotic signals (Harrison, 2005; Akiyama et al., 2005; Besserer et al., 2006).

Advantageously, the lipochitooligosaccharides of the invention are present in the composition at a concentration of between 10^{-5} M and 10^{-10} M. When added to a culture medium for production of AM fungal spores, they are present at a concentration comprised between 10^{-6} M and 10^{-11} M in the medium.

The invention will be understood more clearly with the aid of the additional description which refers to the examples below and to the appended drawings. It should be

clearly understood, however, that these examples and drawings, are given solely as illustration of the subject of the invention and do not constitute in any manner a limitation thereof.

LEGENDS OF THE DRAWINGS

Fig. 1. Biological assays used to detect AM fungal symbiotic signals

a. The MtENOD11 assay. Roots of transgenic *M. truncatula* Jemalong A17 seedlings carrying the reporter construct p*MtENOD11-GUS*. GUS activity is detected by histochemical staining with 5-bromo-4-chloro-3-indolyl-b-glucuronic. (1) Control roots treated with acetonitrile 2.5%. (2) Fraction after SPE and elution with 50% acetonitrile diluted 40 times. (3) The same fraction with a further ten-fold dilution.

b. The VsHab assay. Root hairs of vetch (*Vicia sativa* subsp. *nigra*) observed under light microscope after staining with methylene blue. (1) Root hairs treated with an inactive fraction are straight. (2) Root hairs treated with active fractions are clearly branched.

Fig. 2. Semi-preparative C18 reverse phase HPLC profile of extracts from mycorrhized root exudates.

The initial isocratic phase with 20% acetonitrile lasted 10 min and was followed by a 20-100% acetonitrile gradient for 20 min. The profile reveals the abundance of contaminating material present in mycorrhized root exudates. Fractions were collected every two minutes and were tested for biological activity on MtENOD11 and VsHab. Horizontal bars indicate the retention time of compounds in fraction A that are active on MtENOD11, and of compounds in fraction B, more hydrophobic, that are active on VsHab.

Fig. 3. Semi-preparative C18 reverse phase HPLC profile of extracts from germinating spore exudates.

The chromatographic conditions are the same as in Fig. 2. The profile reveals that spore exudates contain much less contaminating material than mycorrhized root exudates. Fractions were collected every two minutes and were tested for biological activity on MtENOD11 and VsHab. Horizontal bars indicate the retention time of compounds in fraction A that are active on MtENOD11, and of compounds in fraction B, more hydrophobic, that are active on VsHab.

Fig. 4. Influence of mild methanolic hydrolysis on the biological activity of fraction A.

Mild methanolic hydrolysis has been reported to remove the sulfate moiety of sulfated LCOs without altering other structural features of these molecules. Fraction A collected during semi-preparative HPLC of germinating spore exudates was mildly hydrolyzed and tested for biological activity on MtENOD11 and VsHab assays. Biological activity is represented by vertical bars. Whereas unhydrolyzed fraction A is active on MtENOD11 and inactive on VsHab, the hydrolyzed fraction has lost activity on MtENOD11

and gained activity on VsHab. These data indicate that the biological activity of fraction A on the MtENOD11 assay is due to the presence of sulfated LCOs.

Fig. 5. Tetrameric sulfated LCOs N-acylated by C16 fatty acids.

UPLC/MS traces, in the negative mode, of the fraction 4 isolated after semi-preparative C18 HPLC. Extracted ion currents corresponding to sulfated tetramers and corresponding spectra are given. This figure indicates that compounds responding at m/z 1101.5, 1103.5 and 1105.5 are effectively present in the samples. These m/z correspond to sulfated tetrameric LCOs N-acylated by C16:2, C16:1 and C16:0 respectively. Regarding the relative intensities of the three, it appears that 1105.5 (LCO-IV-C16:0) is the most abundant, followed by 1103.5 (LCO-IV-C16:1).

Fig. 6. Tetrameric sulfated LCOs N-acylated by C18:1 fatty acid.

UPLC/MS traces, in the negative mode of the fraction 5 isolated after semi-preparative C18 HPLC showing that the most abundant compound (m/z 1135.5) is N-acylated by a C18:1 fatty acid.

This profile also indicates that no LCO bearing a C18:0 fatty acid is present in this fraction (m/z 1133.5) as this ion is only the isotope +2 of the LCO bearing the C18:1 chain. As the second mass spectrum demonstrates, the di-unsaturated C18-LCO is a very minor compound.

Fig. 7. Pentameric sulfated LCOs N-acylated by C18:1 fatty acyl.

This profile shows that lipochitopentamers are also present, but compared to the corresponding tetramers (see Fig. 5) they are approximately 30 times less abundant. The LCO-V-C18:1 can be detected.

Fig. 8. Checking for the presence or absence of a given compound.

When the requested mass does not correspond to ions present in the sample the profile instead of giving a single peak gives a very large number of background peaks. The very complex profile obtained with ion current m/z 1332.6 demonstrates the absence of a C18:2 chitopentamer in the sample. In contrast, the clear single peak observed with ion current m/z 1334.6 clearly shows the presence of a C18:1 pentamer.

Fig. 9. Comparison of fragmentation pattern by MS/MS of major sulfated Myc factor and *S. meliloti* Nod factor

Demonstrating the presence of compounds having the adequate mass at the expected HPLC retention time, is not sufficient to attest their structure. Therefore, we performed MS/MS analysis of the major sulfated Myc compound. This figure presents the comparison between the *S. meliloti* sulfated tetrameric Nod factor N-acylated by C16:2 in the negative mode MS/MS and the one recorded on the major tetrameric "Myc factor" present in the sample. Characteristic ions of the reducing end at m/z 503 (Y_2), 605 and 706 (Y_3) are clearly detected in both case as well as the characteristic neutral loss of 101 amu (intracyclic

rupture) starting from the molecular ion. The perfect fit between the two fragmentation patterns indicates the structural affiliation of the two molecules.

Fig. 10. Effect of Myc extract fractions on lateral root formation in *M. truncatula*

(A) The AM fungal signal that stimulates LRF is amphiphilic.

Comparison of the effect of aqueous (Aq), butanol (BuOH) and ethyl acetate (EA) extracts of germinating spore exudates (GSP24) on *M. truncatula* A17. The butanol extract stimulates LRF from day 5 on (significant at $P<0.05$), whereas the aqueous and acetyl acetate extracts are not active.

(B) LRF stimulation is mediated via the DMI symbiotic signaling pathway.

Comparison of the effect of mycorrhized root exudate (MRE1) butanol extracts, further purified by SPE eluted with 50% acetonitrile, on *M. truncatula* wild-type (A17) and on a *dmi1* mutant (Y6). The Myc extract stimulates LRF on the wild-type but not on the *dmi1* mutant.

(C) Both fractions A and B stimulate LRF.

Fractions A et B were collected after semi-preparative HPLC of mycorrhized root exudates (MRE-1). Fraction MRE-1 A contained sulfated LCOs and fraction MRE-1 B contained non-sulfated LCOs. Both fractions stimulated LRF significantly ($P<0.05$).

MATERIALS AND METHODS

Sources of Myc Factors

The AM fungus *Glomus intraradices* strain DAOM 197198, which has been maintained in co-culture with excised roots for many years (Chabot et al., 1992), is well characterized and its genome is being sequenced. This strain is used by PREMIER TECH company for the industrial preparation of commercial inoculants and for the production of purified spores for research purpose. For example, these purified spores were used as a source of DNA for the project of *G. intraradices* genome sequencing. We used two sorts of exudates, both purchased from PREMIER TECH 2000 Ltée (Rivère-du-Loup, Québec, Canada):

(i) Exudates from mycorrhized roots (EMR). Mycorrhiza production was achieved by co-cultivation of *G. intraradices* with excised transformed roots of carrot. The growth medium was solidified with Phytagel. After appropriate growth of the mycorrhized roots, the gel was liquefied by adding sodium citrate as chelating agent, and the liquid EMR was conditioned in 4 liter containers which were stored at 4°C.

(ii) Purified sterile spores of the AM fungus *Glomus intraradices* were conditioned by bottles containing approximately one million of spores. Bottles were stored at 4°C. Spores were germinated at 30° C in a 2% CO_2 incubator for 10 days.

Bioassays

To detect the presence of AM fungal symbiotic signals during the various steps of extraction and purification, three bioassays were used. (i) The *M. truncatula ENOD11::GUS* construct was shown to be induced during mycorrhiza formation and by a diffusible compound from diverse AM fungi (Journet et al., 2001; Kosuta et al. 2003) (= MtENOD11 assay). (ii) The lateral root formation in *M. truncatula* was shown to be stimulated by a diffusible compound from diverse AM fungi and the response required the DMI symbiotic signaling pathway (Olah et al., 2005) (= MtLRF assay). (iii) In addition we used a modified *Vicia sativa* (vetch) root hair branching assay which allows the detection of various non-sulfated LCOs (= VsHab assay).

(i) Induction of the symbiotic *MtENOD11* gene in transgenic *Medicago truncatula.*

We have previously shown by experiments in which the AM fungus was separated from the plant root by a cellophane membrane that a diffusible AM fungal compound can induce the expression of an *MtENOD11* promoter-*gusA* transgene in growing lateral roots of *M. truncatula* (Kosuta et al., 2003). The protocol used was as previously described (Andriakaja et al., 2007) with the following modifications: no paper disc was inserted on the top of the agar plate and the treatment was made by addition of 40 microliters per seedling. To check whether the *ENOD11* response was induced via the DMI signaling pathway, we compared the response observed in the *M. truncatula* wild-type line A17 and in a mutant line carrying a mutation in the *DMI1* gene (Y6 mutation).

(ii) Stimulation of lateral root formation in *M. truncatula.*

We have previously shown that a diffusible factor from AM fungi stimulates the formation of lateral roots in *M. truncatula* via the DMI1/DMI2 pathway (Olah et al., 2005). The medium used was as described previously except that vitamins were not added. The wild-type *M. truncatula* Jemalong A17 line and the Y6 *dmi1* mutant were used for the assay.

(iii) Root hair branching of vetch

Vetch (*Vicia sativa* subsp. *nigra*) is a small seeded legume which is convenient for the microscopical observation of root hair deformations. Vetch root hair deformations are elicited not only by the Nod factors of the specific bacterial symbiont *Rhizobium leguminosarum* bv. *viciae* but also by a variety of non-sulfated Nod factors (Roche et al., 1991; Price et al., 1992).

This assay is thus appropriate to detect the presence of non-sulfated LCOs. In previous reports the assay was done in a liquid medium. We have devised an assay on agar plate which is more sensitive and reproducible. Seeds were first sterilized in sulfuric acid for 20 min, rinsed twice with sterile water, and then treated for 20 min in calcium hypochlorite (5g/150ml after paper filtration) and rinsed five times with sterile water. Seeds were left in

water overnight at 4°C, transferred onto soft agar plates and incubated three days at 4°C to increase the homogeneity of germination. Plates were then left for 36 hours at 22°C in the dark for germination. Five young seedlings (root length of approximately 1 cm) were sown in Petri dish, on Fahraeus agar plates, surrounded with parafilm, and left three days, in a vertical position in a growth chamber at 22°C. When roots became hairy, 40 microliters of the solution to be tested were gently deposited along the roots, and seedlings were grown for 30 hours at 22°C. For root hair branching observation, roots were sectioned, inserted between a slide and a cover-slip in a 0.02% methylene blue solution, and observed under a light microscope. Ten plants were observed per treatment.

Biochemical analyses

Liquid /liquid extraction for mycorrhized roots exudates:

In a two liter bulb, 1.6 liter of mycorrhized root exudates was extracted a first time with 400 ml (1/4 of volume) butanol (1-butanol or 2-methyl-1-propanol) and the mixture was left for decantation to get a clear butanol phase with a thin interphase, permitting a good separation of the aqueous and butanol phases (at least six hours). The aqueous phase was then extracted a second time with 350 ml (approximately 1/5 of volume) butanol and left overnight. After this second extraction, the total butanol phase (extraction 1 and extraction 2) was evaporated to a volume of approximately 0.5 liter, which was washed by a liquid/liquid extraction with the same volume of bi-distilled water. The washed butanol phase was evaporated, transferred in a small balloon and dried using a rotary evaporator. The dry extract was then re-dissolved in 5 ml water/acetonitrile (1/1) and filtered on cotton (preliminary washed with chloroform) in a 8 ml glass tube and then dried under nitrogen flux.

Liquid/liquid extraction for germinating spores exudates:

Exudates from one million of germinating spores (approximately 150 ml) were first extracted with 1/3 of volume of ethyl acetate. The mixture was left for decantation to get a thin interphase and a good separation of the aqueous and ethyl acetate phases (at least six hours). The aqueous phase was extracted a second time with 1/3 of volume of ethyl acetate overnight. The aqueous phase was then extracted with butanol (1-butanol or 2-methyl-1-propanol) following the same steps as for the ethyl acetate extraction. The butanol and ethyl acetate phases volumes were reduced to few ml using a rotary evaporator. Each phase was transferred into a 5 ml tube and dried under nitrogen flux.

Purification by solid phase extraction (SPE):

Column preparation: The SPE system was made up of a Chromabond 3ml glass column filled with C18 reverse phase (SUPELCO Discovery DSC-18). A first glass fiber filter was introduced at the bottom of the column. The solid phase was added into the column to represent 3.5 cm height of the column. A second glass fiber filter was laid on top of

the solid phase and pushed in to compress the solid phase. Before use, the column was conditioned with water, then with acetonitrile (ACN) 20% in water.

Pre-filtration: Extract was dissolved in one ml of 20% ACN. The extract was filtered on cotton in a Pasteur pipette (preliminary washed with chloroform) and deposited on the C18 column. The tube and the filters were rinsed with 1.5 ml of ACN 20%.

Chromatography: Using a syringe, the extract was pushed through the C18 phase. The running out liquid of the column was collected in an 8 ml glass tube. To get rid of the non-adsorbed compounds, the phase was abundantly washed (equivalent 5 times the volume of the solid phase with 20% ACN in water). This volume was recovered in the same tube. Then molecules retained on the column were eluted with a 50% solution of ACN in water. The elution volume being equivalent to 5 times the solid phase volume was recovered in a second glass tube. Finally the strongly adsorbed molecules were eluted from the column with 100% ACN. The volume of solvent (about 6 ml) was recovered in a third tube. The three solutions (20%, 50% and 100% of ACN) were evaporated under nitrogen flux, in order to get dry residues. Each residue could then be re-dissolved in the volume appropriate to realize the semi-preparative HPLC. A SPE column was used to purify approximately 5 liters of mycorrhized root exudates.

Semi-preparative HPLC:

Purification was performed on a High-Performance Liquid Chromatography Shimadzu LC10 separation module (Shimadzu corporation, Kyoto, Japan) with a semi-preparative C18 reverse phase column (8 mm x 250 mm; 5 µm, Equisorb, CIL-Cluzeau). The injection loop had 100 microliter volume. The chromatographic procedure was the following: for 10 min in isocratic mode with solvent A (20% acetonitrile in water), followed by a linear gradient for 20 min from solvent A to solvent B (100% acetonitrile) and another isocratic step at 100% acetonitrile for 5 min. Two minutes are necessary to come back to the initial conditions (20% ACN). The flow rate was 2 ml min^{-1} and UV absorption was monitored at 206 nm. Collection of samples along the gradient was done every minute (2 ml) resulting in 14 fractions.

UPLC-ToF MS analyses:

Each fraction collected from semi-preparative HPLC was submitted to UPLC-MS analysis on an Acquity UPLC coupled to Q-Tof Premier mass spectrometer (Waters Corporation). The UPLC column was an Acquity column (2.1 mm x 10 cm, 1.7µm)(Waters, USA), and the flow rate was 0.45 ml/min. For the more hydrophilic compounds (semi-preparative HPLC fractions 1 to 9) the program was a linear gradient ranging from 10% ACN (in 1% acetic acid/water) to 100% ACN within 7 minutes, followed by an isocratic step at 100% ACN for 2 min and then a return to the initial conditions (2 min) and a reconditioning step of 1 min with 10% ACN (in 1% acetic acid/water). To get a better resolution of the more hydrophobic compounds (semi-preparative HPLC fractions 6 to 11) the

UPLC gradient was more extended: linear gradient starting at 25% ACN in 0.1% acetic acid/water and reaching 100% ACN within 7 minutes. For the mass spectrometer, capillary was set to 3.2 kV and the cone to 10 V. Internal lock mass was performed by continuous introduction into the source of a Leucine-enkephalin solution. Spectrometer was calibrated before each experiment. The more hydrophilic compounds (semi-preparative HPLC fractions 1 to 9) were analyzed in both the negative and positive modes in order to facilitate respectively the detection of charged (sulfated) and uncharged (non-sulfated) compounds.

For fragmentation of molecules, specific ions were selected and submitted to MS/MS analysis using collision energy at 15V.

Mild hydrolysis:

This method is used to remove the sulfate moiety of sulfated LCOs without affecting the rest of the molecules (Roche et al., 1991b). Fraction A, eluting between 15 and 16 min on semi-preparative HPLC, was transferred in a screw glass vial and dried under nitrogen flux. It was re-dissolved two times in anhydrous methanol and dried again, in order to remove residual water. 250 µl of 0.05M HCl in methanol was added to the dry sample. The reaction was carried out overnight at room temperature. The sample was then dried again under nitrogen flux and washed twice with anhydrous methanol, in order to remove all the acid.

RESULTS

EXAMPLE 1: PURIFICATION OF MYC FACTORS FROM MYCORRHIZED ROOTS EXUDATES AND FROM GERMINATING SPORES EXUDATES

General strategy

Glomus intraradices strain DAOM 197198 was used as a source of Myc factors because this strain has a broad host-range and is used for large-scale industrial production of AM fungi inoculants. This strain is well characterized and its genome is being sequenced. Two sources of Myc factors were used in a complementary manner. Exudates from mycorrhized roots have the advantage of permitting extraction from large volumes with the possibility of obtaining significant amounts of Myc factors. The disadvantage of this source is that exudates contain a mixture of compounds from both plant and fungal origin. This is why we also used another source, exudates from purified germinating spores, which contain only compounds of AM fungal origin but have the disadvantage of producing extremely low concentrations of Myc factors.

Biologically active compounds present in AM fungal exudates are amphiphilic

Mycorrhized root exudates were first extracted by a liquid-liquid procedure, with butanol and ethyl acetate. Aqueous, butanol and ethyl acetate phases were checked for biological activity with MtENOD11 and VsHab bioassays: activity was found in the butanol fraction which indicated that Myc factors are amphiphilic compounds. As shown in Fig.1, an

active compound could be detected with the MtENOD11 assay by a blue staining appearing on the growing roots, and with the VsHab assay by the appearance of clear branches close to the tip of vetch root hairs.

Then the butanol extract was submitted to Solid Phase Extraction (SPE) with a reverse phase C18 column and successively eluted with 20%, 50% and 100% acetonitrile solvent. Biological activity was found in the fraction eluted by 50% acetonitrile on MtENOD11 and VsHab assays confirming that the active compound(s) is amphiphilic. Similar results were obtained with more than five independent samples of mycorrhized root exudates. A very slight activity on VsHab could also be observed sometimes in the 100% acetonitrile eluate, suggesting that different compounds could be responsible for the MtENOD11 and the VsHab responses, the compound acting on the VsHab assay being slightly more hydrophobic than the compound acting on the MtENOD11 assay.

Exudates of germinating spores were extracted by the same liquid-liquid procedure with butanol and ethyl acetate. The activity on both MtENOD11 and VsHab assays was present only in the butanol phase. The same results were obtained with five independent samples of germinating spores. We can thus conclude that the amphiphilic compound(s) active on the MtENOD11 and VsHab assays are of AM fungal origin.

Two types of active compounds in mycorrhized root exudates

To further resolve the compounds active on MtENOD11 and VsHab and get information on their chromatographic properties, the butanol fraction had to be analyzed by HPLC. However, the mycorrhized root exudates being highly contaminated by plant root compounds and by phytagel, the butanol fraction was pre-treated by SPE before the HPLC step, as described in the preceding paragraph. The SPE fraction eluted by 50% acetonitrile, and active on the two bioassays, was then analyzed in a semi-preparative HPLC with a reverse phase C18 column and an acetonitrile-water gradient. Fourteen fractions were collected every two minutes. A typical profile is given in Fig. 2. Each fraction was tested for activity on MtENOD11 and VsHab. Fractions eluted at 30-48% of acetonitrile (ACN) (fraction A) were found to be active on MtENOD11, and fractions eluted at 64-72% of ACN (fraction B) were active on the VsHab bioassay. These data show that it is not the same compound which is active on both bioassays. The compound(s) active on MtENOD11 is more hydrophilic than the one(s) active on VsHab.

It is interesting to note that the elution characteristics of the fraction active on MtENOD11 correspond well to those observed with sulfated Nod factors of *Sinorhizobium meliloti* and *Rhizobium tropici* (33-45%), which can also exhibit activity with the MtENOD11 bioassay. On the other hand the elution characteristics of the fraction active on VsHab correspond well to those observed with non-sulfated Nod factor of *R. leguminosarum* bv. *viciae* (67%) and non-acetylated Nod factors of *Rhizobium meliloti nodHnodL* (66%) which can also exhibit activity with the vetch bioassay. These data are compatible with the

hypothesis that the mycorrhized root exudates contain a mixture of sulfated and non-sulfated LCOs.

Two types of active compounds in germinating spores exudates

Butanol extracts from germinating spores exudates were analyzed in a semi-preparative HPLC in the same conditions as described above. As seen on Fig. 3, spore exudates also contained two types of active compounds, one more hydrophilic active on MtENOD11 (fraction A) and one more hydrophobic active on VsHab (fraction B). The elution characteristics of the two compounds are identical to those observed with the two active compounds from the mycorrhized root exudates. These results indicate that the two active compounds present in the mycorrhized root exudates are of AM fungal origin. Their chromatographic behavior and their biological activities are compatible with the hypothesis that they could correspond to sulfated and non-sulfated LCOs.

Modification of activity associated to desulfatation of fraction A

A mild methanolic hydrolysis of sulfated LCOs, as *S. meliloti* Nod factors, has been shown to remove the sulfate group without causing other structural modifications (Roche et al., 1991b). To check whether the biological activity on MtENOD11 of the fraction A collected after HPLC of butanol extracts from germinating spores exudates could be due to a sulfated LCO, a sample of fraction A was submitted to this mild hydrolysis treatment. The treated fraction totally lost activity on MtENOD11 (see Fig. 4). Interestingly, whereas the fraction A was not originally active on the VsHab bioassay, the treated fraction exhibited a clear activity on VsHab (Fig. 4). That fraction A can after mild hydrolysis gain a function, the activity on VsHab, shows that this very mild methanolic hydrolysis has not drastically degraded the active compound of fraction A, but has simply modified it, probably by the removal of the sulfate moiety which is a very labile O-substitution in LCOs. These data indicate that the activity of fraction A on MtENOD11 could be due to sulfated LCO(s) and that the activity of the more hydrophobic fraction B on VsHab could be due to non-sulfated LCO(s).

EXAMPLE 2: BIOCHEMICAL CHARACTERIZATION OF MYC FACTORS

LC/MS and UPLC/MS

The different fractions obtained after the semi-preparative reversed phase HPLC of mycorrhized root extracts were individually chromatographed on an analytical reversed phase column under ultra-high pressure (UPLC). The detection occurred through ESI-MS.

Results are shown on Figures 5 to 8. These figures present the ion currents corresponding to LCOs supposed to be present in the samples, according to the HPLC and UPLC retention times, and biological activity. If there are compounds exhibiting the requested mass within their isotopic distribution, then they will appear on the chromatogram

as peaks. As the peaks obtained in this manner could be artefactual (peaks might correspond to a minor compound of the isotopic profile), the corresponding spectra are also given in the lower part of each figure.

The first eight HPLC fractions, for which chromatographic behavior and biological activities suggested the presence of sulfated LCOs, were analyzed in the negative mode. According to the retention times measured in UPLC using standard tetrameric (DP4) and pentameric (DP5) LCOs, exact masses (error less then 10 ppm) corresponding to sulfated DP4 and DP5 entities were searched in the fraction 4 which showed the highest activity on the MtENOD11 assay. In this fraction, masses corresponding to sulfated DP4 bearing C16 acyls could easily be detected (Fig. 5). In agreement with their respective HPLC retention times, we were able to detect in the previous fraction (fraction 3) the corresponding DP5s (Fig. 7) and in the following (fraction 5) sulfated DP4 bearing C18 chains (Fig. 6). In the next fractions (6 to 8) DP3 entities have been researched without success. The compounds have been characterized first using different ion currents (fit between the called exact masses and the expected retention times) and secondly regarding the isotopic profile of the corresponding spectra. Fig. 8 illustrates the efficiency of the method to detect the presence or absence of a given compound, having a specific mass. Ion current m/z 1332.6, corresponding to a putative LCO(V,C18:2,S) produced only amplification of background noise (no individual well-defined peak), whereas the ion current m/z 1334.6 corresponding to a putative LCO(V,C18:1,S) clearly demonstrated the presence of a UPLC peak containing a compound exhibiting the expected mass (data confirmed by the recorded mass spectrum). Thus the Myc extracts contain the pentameric sulfated LCO N-acylated by C18:1 but no derivative acylated by C18:2.

By using this procedure it was not possible to detect sulfated DP4 or DP5 entities carrying O-substitutions as acetyl, carbamoyl or fucosyl groups or N-substitution as a methyl group, that are very frequently found in the lipochito-oligosaccharidic Nod factors produced by diverse rhizobial strains.

Fractions 7 to 11 have been analyzed then on UPLC but the detection occurred in the positive ESI-MS mode. The same strategy was applied: ion call followed by analysis of the corresponding spectra.

Mycorrhized root extracts were also analyzed by LC/MS. Fractions eluting between 20 and 23 minutes on the semipreparative HPLC were pooled, dried under nitrogen flux and redissolved in 150 µl of 50% ACN in water and 1% acetic acid. Solutions were directly infused into the ESI source of a Q-Tof Ultima spectrometer (Waters, US). Capillary was set at 3 kV, the cone voltage at 70V, the Rf lens at 35V. In the positive mode the molecular ions of two minor compounds at m/z 1045 and 1047 could be detected corresponding to the sodium cationized tetrameric LCOs bearing an C16:1 and C16:2 acyl and no O-substitution. Masses and isotopic profiles confirmed the proposed structures.

MS/MS

Demonstrating the presence of compounds having the adequate mass at the expected HPLC or UPLC retention time, is not sufficient to attest their structure. Therefore, we performed MS/MS analysis of one of the putative LCO compound. Fig. 9 presents the comparison between the *S. meliloti* sulfated Nod factor DP4 C16:2 in the negative mode MS/MS and the one recorded on the "Myc factor" candidate present in the sample, LCO(IV,C16:0,S). Characteristic ions of the reducing end at m/z 503 (Y_2), 605 and 706 (Y_3) are clearly detected in both case as well as the characteristic neutral loss of 101 amu (intracyclic rupture) starting from the molecular ion. The perfect fit between the two fragmentation patterns indicates the structural affiliation of the two molecules and indicates that the sulfate group is located on the reducing glucosamine residue whereas the fatty acyl substitution is localized on the terminal non-reducing glucosamine residue. Since fragmentation does not produce beta-elimination ions (fatty acid ions) it is very likely that the fatty acyl substitution is an amide on the N atom of the glucosamine residue.

EXAMPLE 3: STIMULATION OF LATERAL ROOT FORMATION BY MYC FACTORS

Butanol extract from mycorrhized root exudates, after further purification by solid phase extraction (SPE) and elution by 50% acetonitrile was incorporated into M plates and tested for growth of *M. truncatula* A17 seedlings. This purified Myc extract induced a significant stimulation of lateral root formation (P = 0.05). When tested on a *M. truncatula dmi1* mutant (Y6) this Myc extract did not stimulate lateral root formation, indicating that this semi-purified extract contained a Myc signal which activates lateral root formation (LRF) via the DMI symbiotic signaling pathway (Fig. 10A).

Germinating spores exudates were extracted with ethyl acetate and butanol. The three extracts (aqueous, ethyl acetate and butanol) were then checked for LRF stimulation on *M. truncatula* A17 seedlings. The butanol extract stimulated LRF significantly (P = 0.05) whereas the aqueous and ethyl acetate extracts were not active (Fig.10B). This experiment confirms the AM fungal origin of the amphiphilic compound(s) eliciting LRF stimulation.

The butanol extract of mycorrhized root exudates, after SPE, was further purified by semi-preparative HPLC and the fractions corresponding to sulfated LCOs (fraction A, active on the MtENOD11 assay) and to non-sulfated LCOs (fraction B, active on the VsHab assay) were collected separately and tested on *M. truncatula* A17 seedlings. The two fractions were found to significantly stimulate LRF (Fig. 10C). These data indicate that the Myc factors are made of a mixture of sulfated and non-sulfated simple LCOs that are both able to stimulate the formation of lateral roots in plants.

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CLAIMS

1) A process for obtaining lipochitooligosaccharides from a fungus of the group Glomeromycota, characterised in that it comprises obtaining exudates from plant roots mycorrhized with said fungus, or from germinating spores of said fungus, extracting said exudates with butanol, and recovering the butanol extract containing said lipochitooligosaccharides.

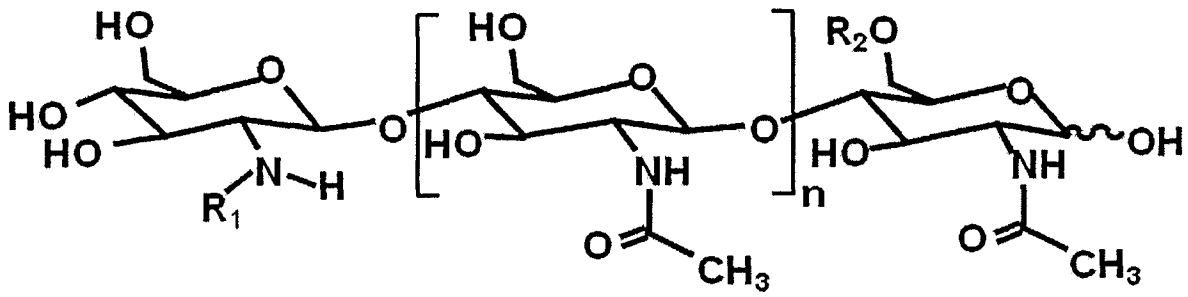
2) A process of claim 1, characterised in that it further comprises the steps of subjecting said butanol extract to reverse-phase liquid chromatography on a C18 column, with successive washes at 20%, 50% and 100% acetonitrile and recovering the fraction eluted at 50% acetonitrile containing said lipochitooligosaccharides .

3) A process of claim 2, characterised in that it further comprises the steps of subjecting said fraction eluted at 50% acetonitrile to reverse-phase high-performance liquid chromatography on a C18 column, using a linear gradient of 20% to 100% acetonitrile, and recovering the fraction eluted at 30-48% of acetonitrile which contains sulfated lipochitooligosaccharides, and/or the fraction eluted at 64-72% of acetonitrile which contains non-sulfated lipochitooligosaccharides.

4) A process of any of claims 1 to 3 characterised in that said fungus from the group Glomeromycota is *Glomus intraradices*.

5) A lipochitooligosaccharide preparation obtainable by a process of any of claims 1 to 4.

6) A lipochitooligosaccharide preparation of claim 5, which comprises a lipochitooligosaccharide defined by the formula (I) below:



(I)

wherein n = 0, 1, 2, 3, 4, or 5, R_1 represents a fatty acid chain containing 14 to 20 carbon atoms, which can be saturated, or mono-, di-, tri- tetra-, penta-, or hexaunsaturated, and R_2 represents H or SO_3H.

7) The use of a lipochitooligosaccharide preparation of any of claims 5 or 6 to stimulate mycorrhization of a plant.

8) The use of a lipochitooligosaccharide preparation of any of claims 5 or 6 to stimulate the root system development of a plant.

9) The use of a lipochitooligosaccharide preparation of any of claims 5 or 6 as an additive for production of an arbuscular mycorrhizal inoculant.

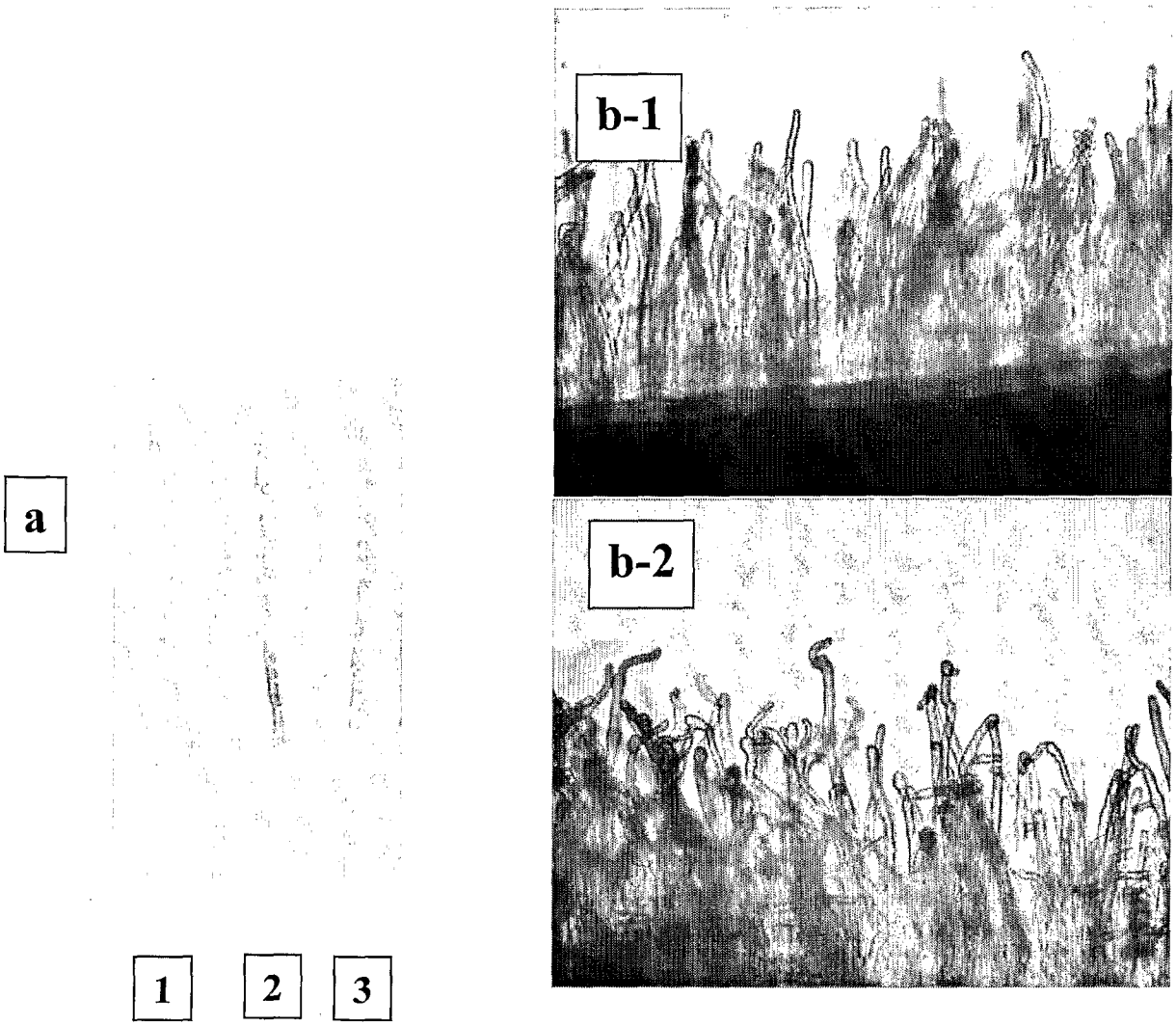


Figure 1

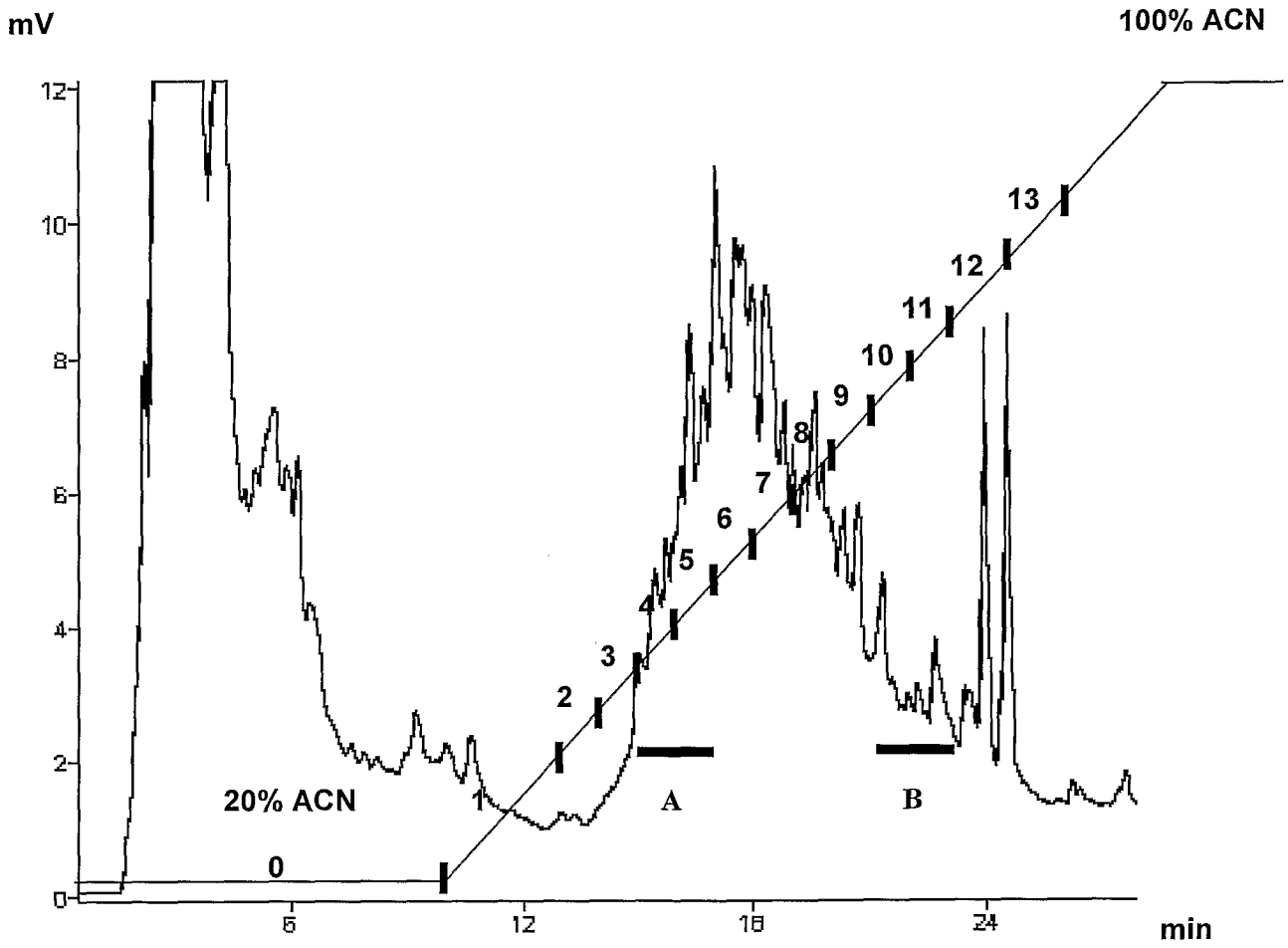

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Figure 2

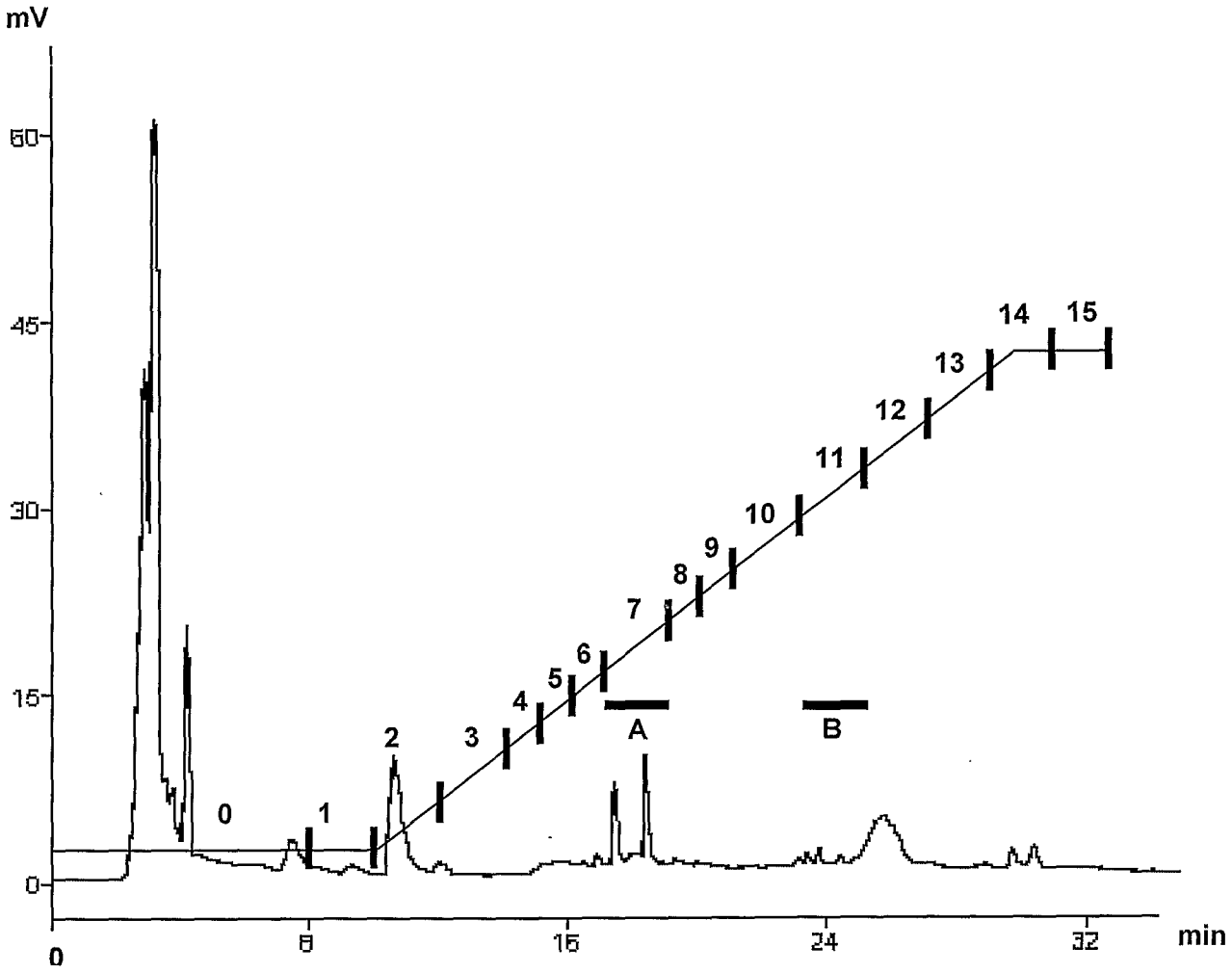
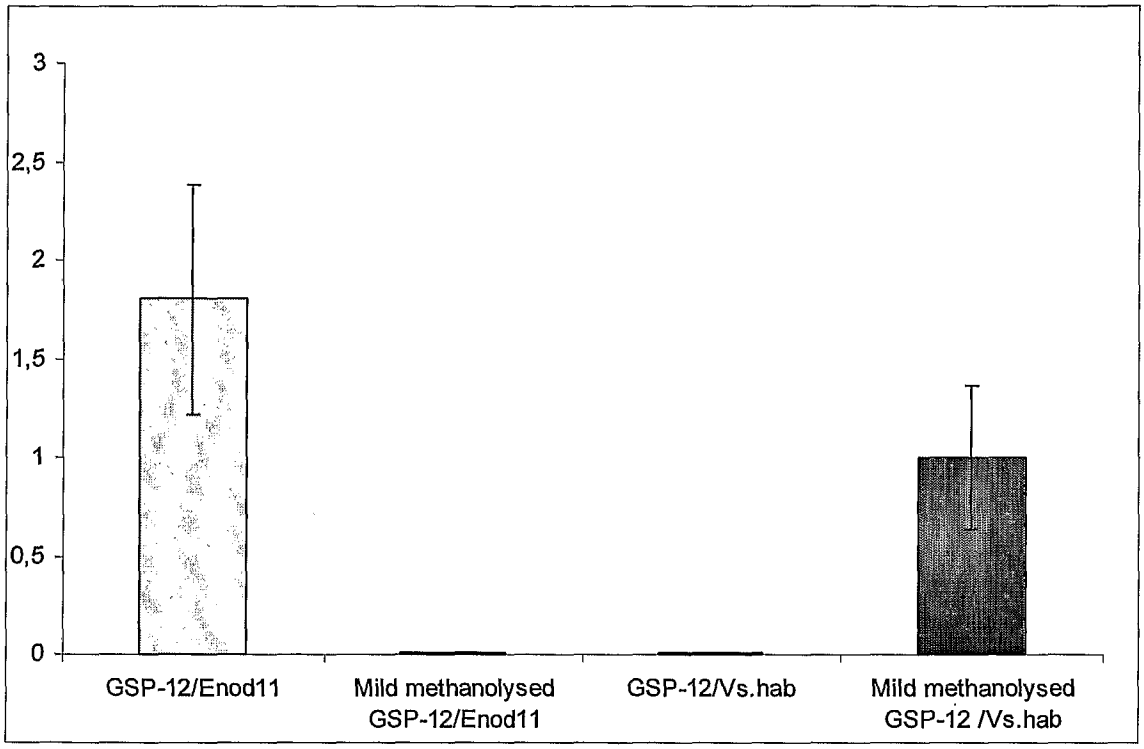


Figure 3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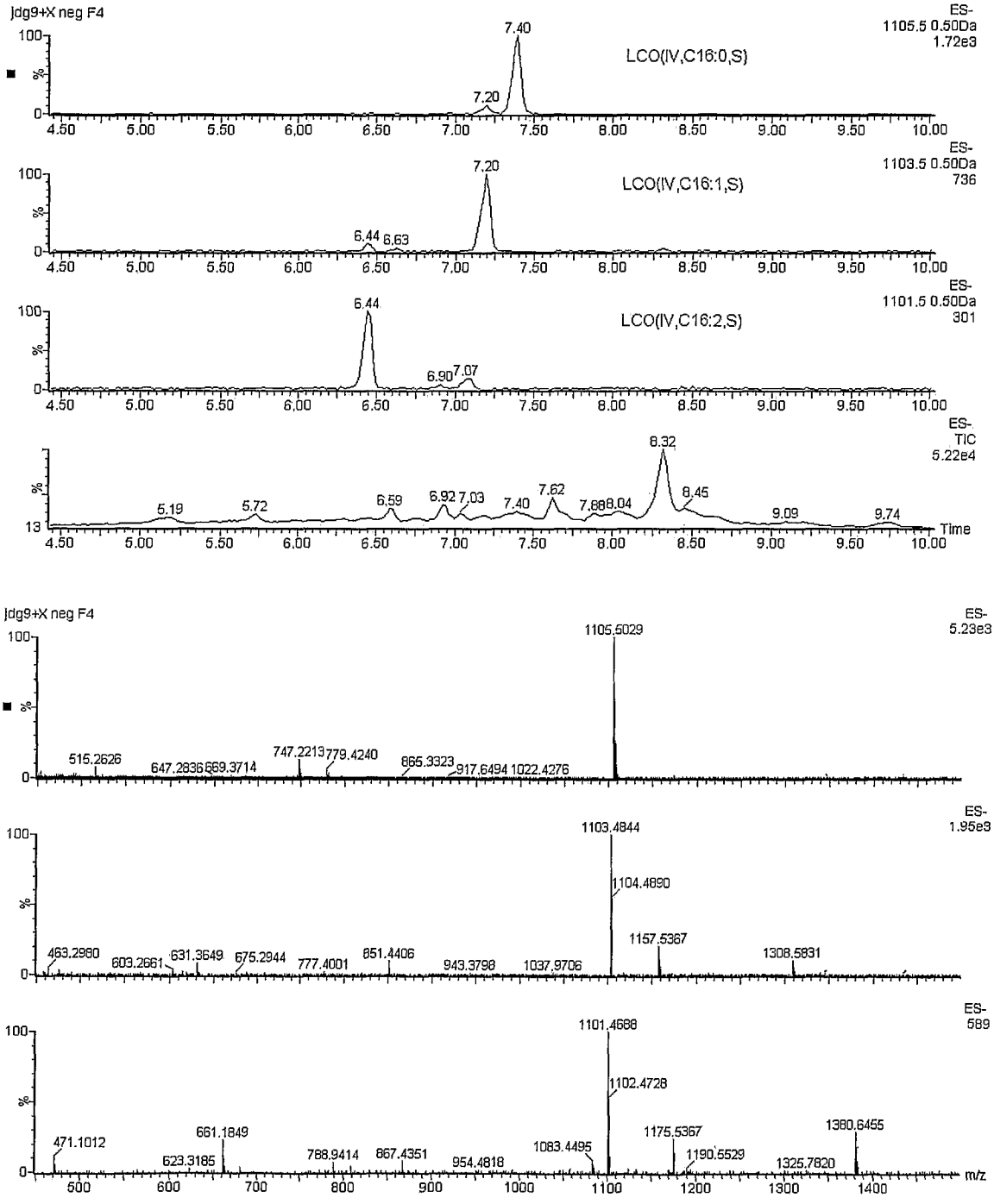
GSP-12/Enod11
Mild methanolysed GSP-12/Enod11
GSP-12/Vs.hab
Mild methanolysed GSP-12 /Vs.hab

Figure 4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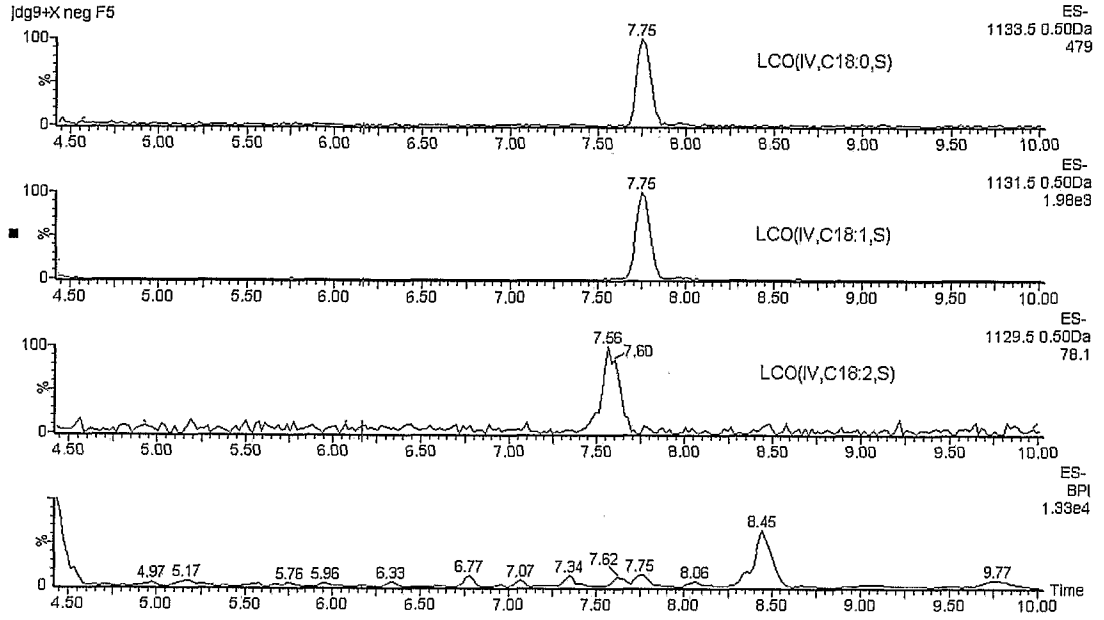
1103.5 0.50Da
736
LCO(IV,C16:1,S)
7.20
6.44
6.63
ES-
1101.5 0.50Da
301
LCO(IV,C16:2,S)
6.44
6.90
7.07
ES-
TIC
5.22e4
8.32
5.19
5.72
6.59
6.92
7.03
7.40
7.62
7.88
8.04
8.45
9.09
9.74
Time
jdg9+X neg F4
ES-
5.23e3
1105.5029
515.2626
647.2836
669.3714
747.2213
779.4240
865.3323
917.6494
1022.4276
ES-
1.95e3
1103.4844
1104.4890
1157.5367
463.2980
603.2661
631.3649
675.2944
777.4001
851.4406
943.3798
1037.9706
1308.5831
ES-
589
1101.4688
1102.4728
471.1012
623.3185
661.1849
788.9414
867.4351
954.4818
1083.4495
1175.5367
1190.5529
1325.7820
1380.6455
m/z

Figure 5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1133.5 0.50Da
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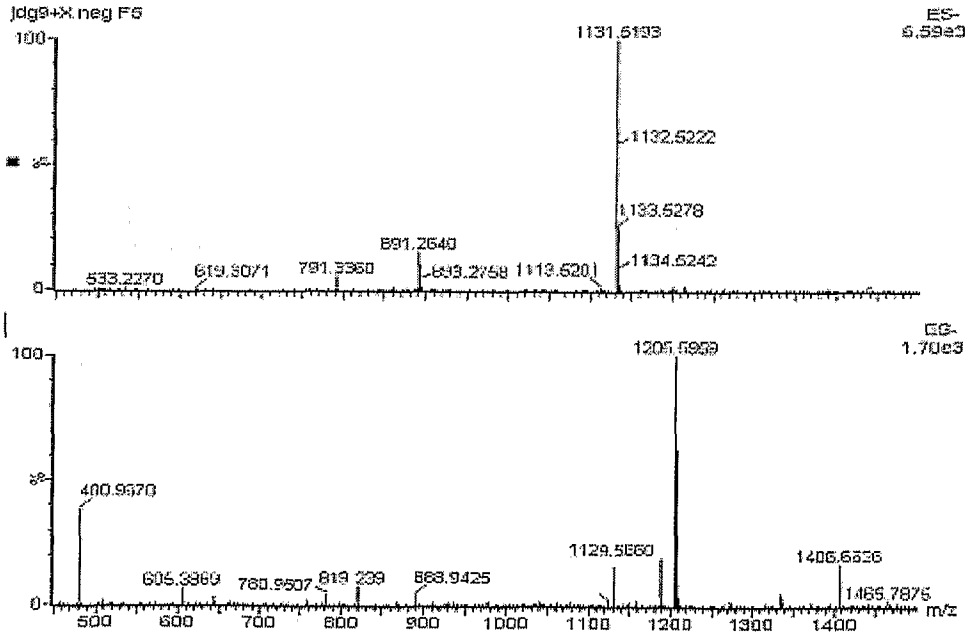
7.75
LCO(IV,C18:0,S)
ES-
1131.5 0.50Da
1.98e3
7.75
LCO(IV,C18:1,S)
ES-
1129.5 0.50Da
78.1
7.56
7.60
LCO(IV,C18:2,S)
ES-
BPI
1.33e4
8.45
4.97 5.17
5.76 5.96
6.33
6.77
7.07
7.34
7.62
7.75
8.06
9.77
Time


jdg9+X neg F5
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1132.5222
1133.5278
1134.5242
891.2640
533.2270
619.3071
791.3360
893.2758
1113.620
ES-
1205.5959
1129.5860
1406.6536
400.9670
605.3369
780.9507
819.239
868.9425
1469.7875
m/z

Figure 6

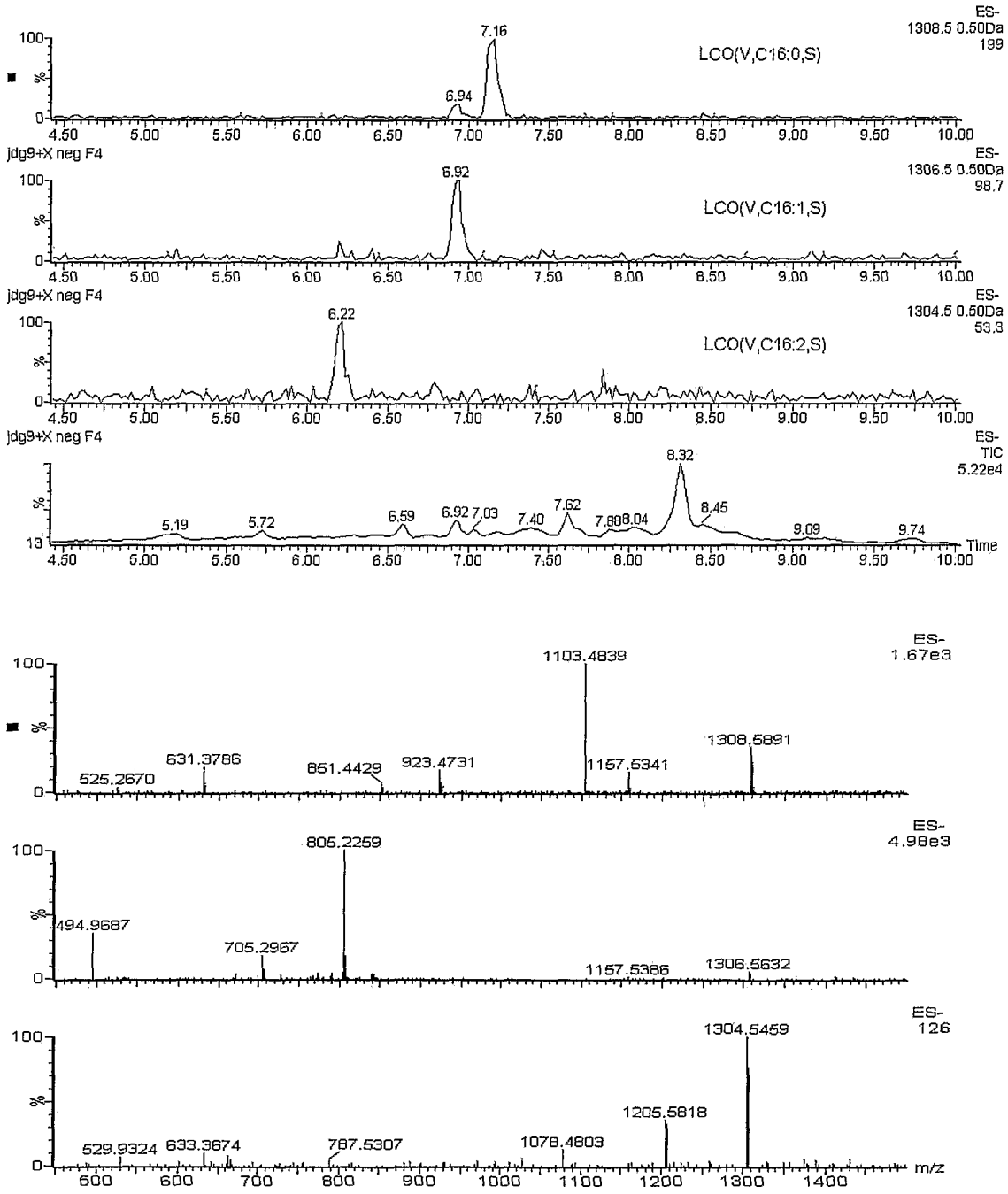
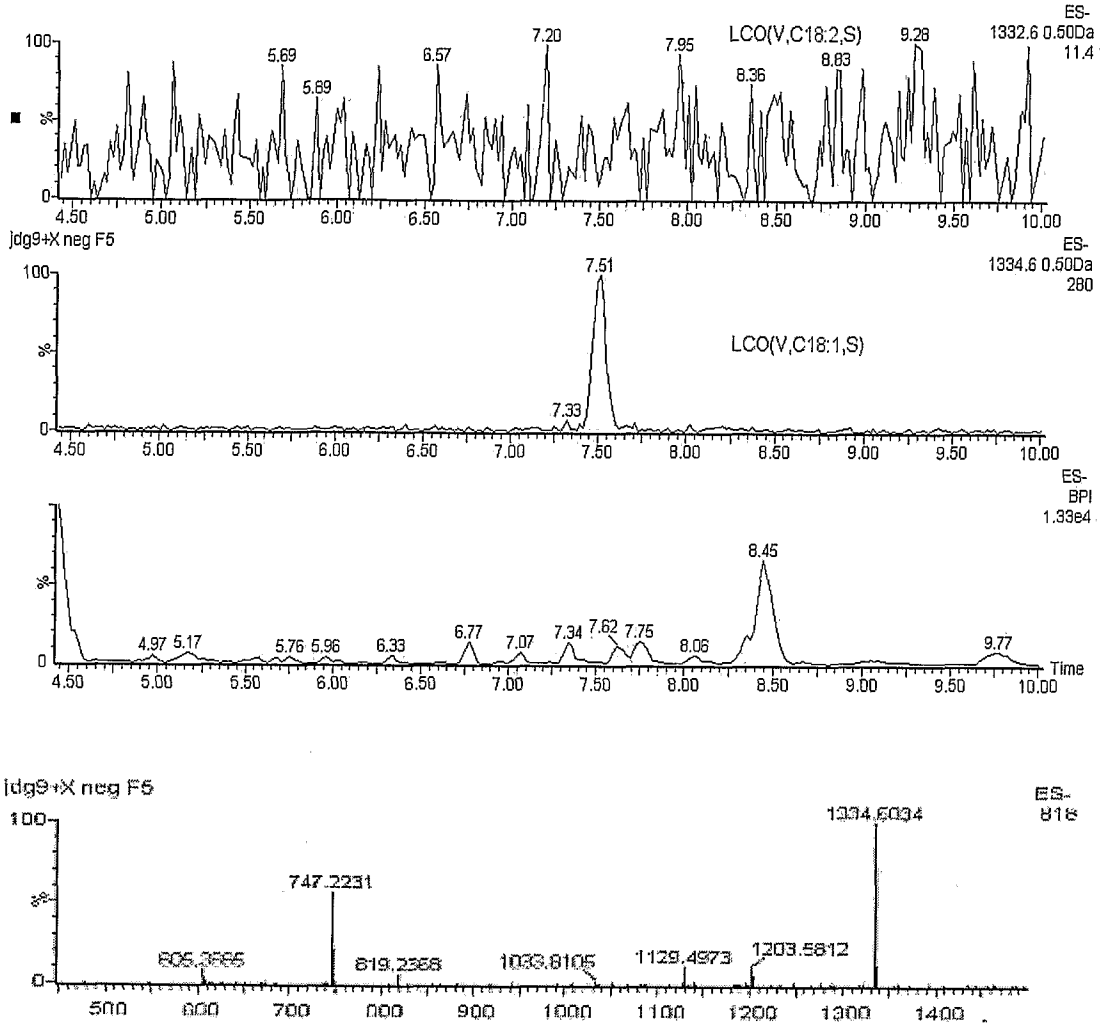

ES-
1308.5 0.50Da
199
LCO(V,C16:0,S)
7.16
6.94
jdg9+X neg F4
ES-
1306.5 0.50Da
98.7
LCO(V,C16:1,S)
6.92
jdg9+X neg F4
ES-
1304.5 0.50Da
53.3
LCO(V,C16:2,S)
6.22
jdg9+X neg F4
ES-
TIC
5.22e4
8.32
5.19
5.72
6.59
6.92
7.03
7.40
7.62
7.88
8.04
8.45
9.09
9.74
Time
ES-
1.67e3
1103.4839
1308.5891
631.3786
525.2670
851.4429
923.4731
1157.5341
ES-
4.98e3
805.2259
494.9687
705.2967
1157.5386
1306.5632
ES-
126
1304.5459
1205.5818
529.9324
633.3674
787.5307
1078.4803
m/z

Figure 7


ES-
1332.6 0.50Da
11.4
LCO(V,C18:2,S)
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5.69
6.57
7.20
7.95
8.36
8.63
9.28
jdg9+X neg F5
ES-
1334.6 0.50Da
280
7.51
7.33
LCO(V,C18:1,S)
ES-
BPI
1.33e4
8.45
4.97
5.17
5.76
5.96
6.33
6.77
7.07
7.34
7.62
7.75
8.06
9.77
Time
jdg9+X neg F5
ES-
818
1334.6094
747.2231
805.3565
819.2368
1033.8105
1129.4973
1203.5812

Figure 8

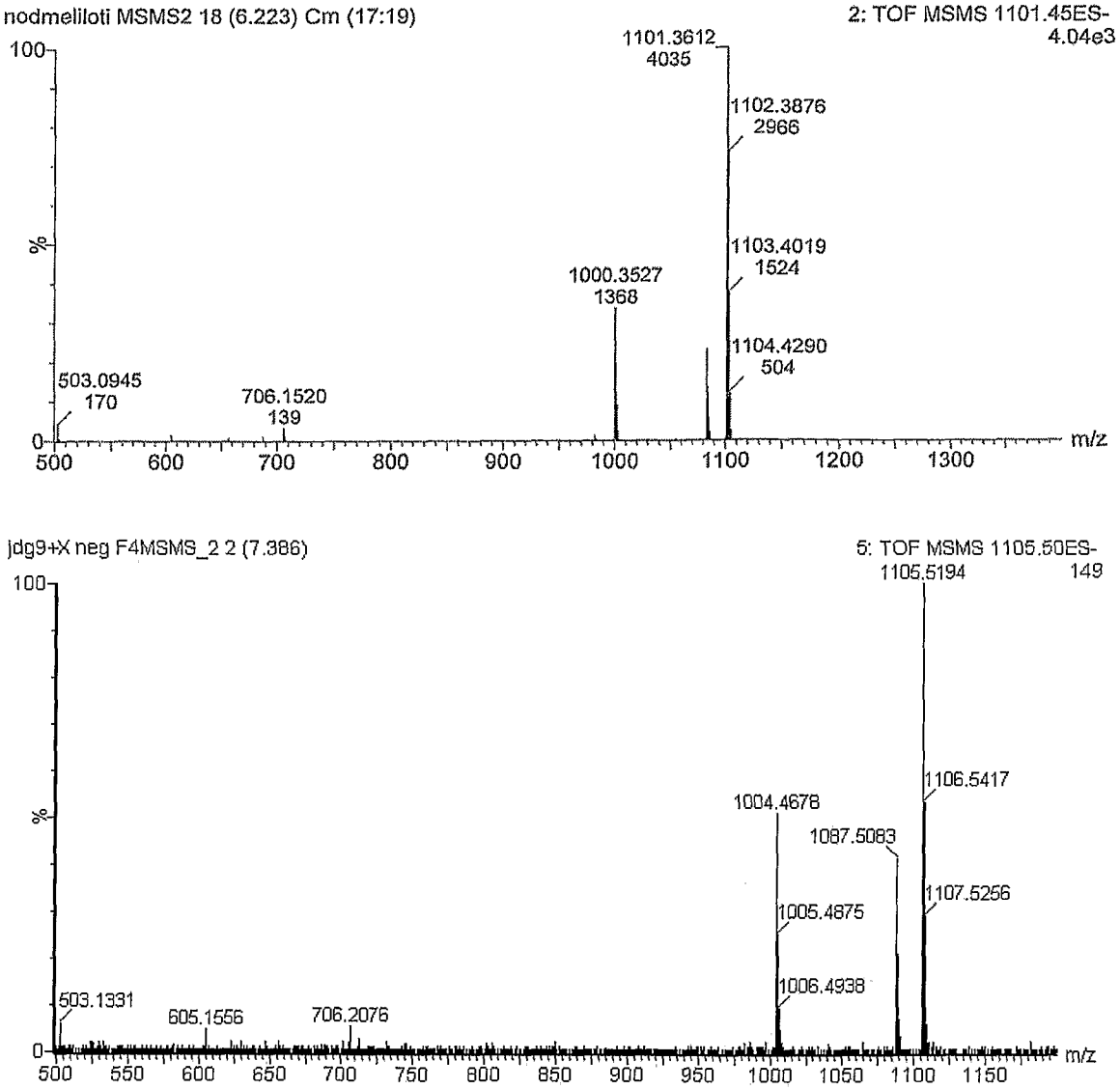
nodmeliloti MSMS2 18 (6.223) Cm (17:19)
2: TOF MSMS 1101.45ES-
4.04e3
100
%
0
1101.3612
4035
1102.3876
2966
1103.4019
1524
1000.3527
1368
1104.4290
504
503.0945
170
706.1520
139
500
600
700
800
900
1000
1100
1200
1300
m/z
jdg9+X neg F4MSMS_2 2 (7.386)
5: TOF MSMS 1105.50ES-
1105.5194
149
100
%
0
1106.5417
1004.4678
1087.5083
1107.5256
1005.4875
1006.4938
503.1331
605.1556
706.2076
500
550
600
650
700
750
800
850
900
950
1000
1050
1100
1150
m/z

Figure 9

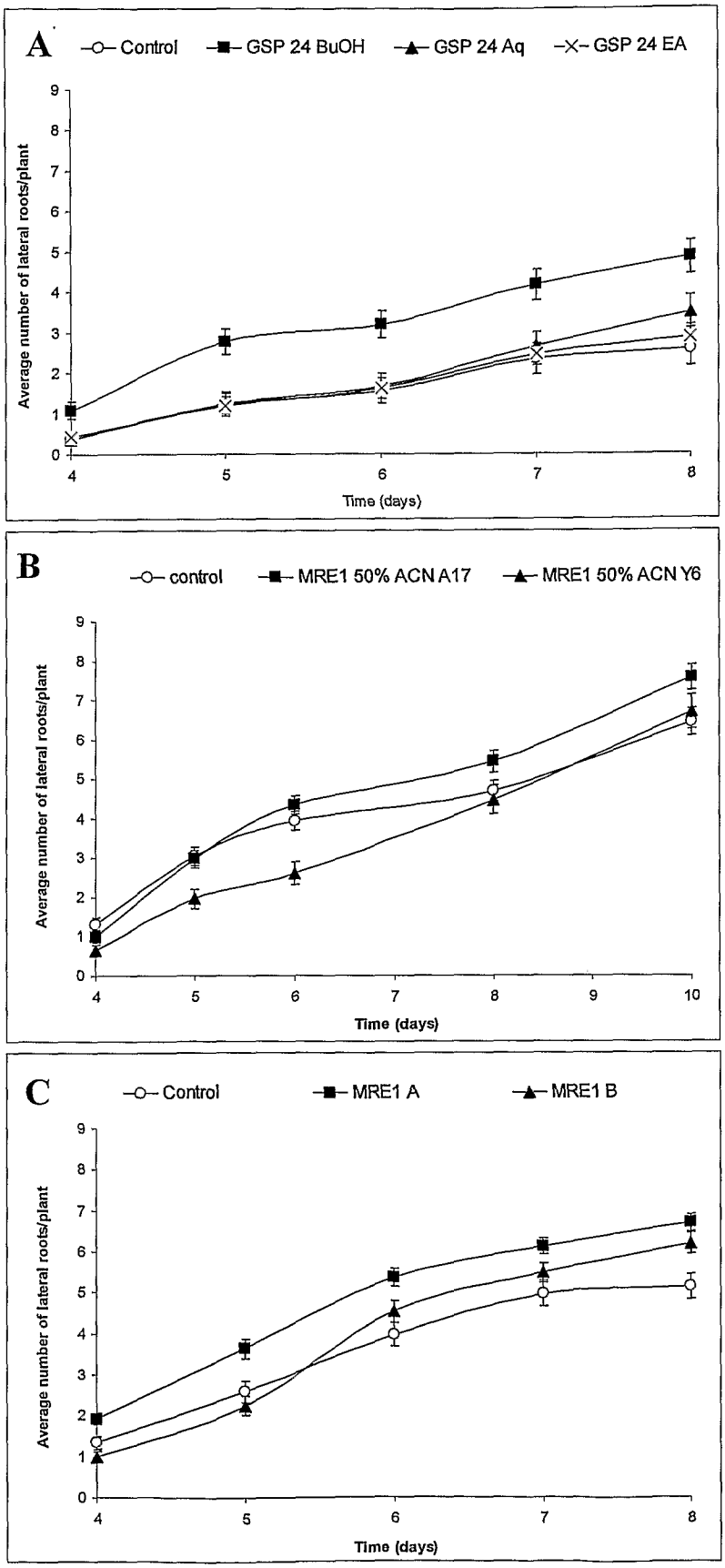
A
Control
GSP 24 BuOH
GSP 24 Aq
GSP 24 EA
Average number of lateral roots/plant
Time (days)
B
control
MRE1 50% ACN A17
MRE1 50% ACN Y6
Average number of lateral roots/plant
Time (days)
C
Control
MRE1 A
MRE1 B
Average number of lateral roots/plant
Time (days)

Figure 10

INTERNATIONAL SEARCH REPORT

International application No
PCT/IB2008/003484

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07H15/12 A01N43/16

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)
C07H A01N

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, 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	PACIOS-BRAS, CRISTINA ET AL: "Novel lipochitin oligosaccharide structures produced by Rhizobium etli KIM5s" CARBOHYDRATE RESEARCH (2002), 337(13), 1193 1202 CODEN: CRBRAT; ISSN: 0008-6215, 2002, XP002543410 Tables	5-9
Y	page 1198	1-4
X	LOPEZ-LARA, ISABEL M. ET AL: "Structural identification of the lipo-chitin oligosaccharide nodulation signals of Rhizobium loti" MOLECULAR MICROBIOLOGY (1995), 15(4), 627 -38 CODEN: MOMIEE; ISSN: 0950-382X, 1995, XP002543411 Figures	5-9
Y	page 635, right-hand column	1-4

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[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents :

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"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
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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
28 August 2009	09/09/2009

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Fax: (+31–70) 340–3016	Klein, Didier

INTERNATIONAL SEARCH REPORT

International application No
PCT/IB2008/003484

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	BOONE, CAROLIEN M. ET AL: "Structural characterisation of lipo-chitin oligosaccharides isolated from Bradyrhizobium aspalati, microsymbionts of commercially important South African legumes" CARBOHYDRATE RESEARCH (1999), 317(1-4), 155-163 CODEN: CRBRAT; ISSN: 0008-6215, 1999, XP002543412 figures 4,5; table 1	5
Y	page 157	1-4
X	COHN ET AL: "Legume nodule organogenesis" TRENDS IN PLANT SCIENCE, ELSEVIER SCIENCE, OXFORD, GB, vol. 3, no. 3, 1 March 1998 (1998-03-01), pages 105-110, XP005030942 ISSN: 1360-1385 figure 1; table 1	5-9
X	JOHN M ET AL: "Cell signalling by oligosaccharides" TRENDS IN PLANT SCIENCE, ELSEVIER SCIENCE, OXFORD, GB, vol. 2, no. 3, 1 March 1997 (1997-03-01), pages 111-115, XP004806822 ISSN: 1360-1385 figure 2	5-9
X	OLSTHOORN, MAURIEN M. A. ET AL: "Novel Branched Nod Factor Structure Results from .alpha.-(1.fwdarw.3) Fucosyl Transferase Activity: The Major Lipo-Chitin Oligosaccharides from Mesorhizobium loti Strain NZP2213 Bear an .alpha.-(1.fwdarw.3) Fucosyl Substituent on a Nonterminal Backbone Residue" BIOCHEMISTRY (1998), 37(25), 9024 -9032 CODEN: BICHAW; ISSN: 0006-2960, 1998, XP002543413 table 1	5-9
X	RENIER A ET AL: "Symbiotic properties of Methylobacterium nodulans ORS 2060<T>: A classic process for an atypical symbiont" SOIL BIOLOGY AND BIOCHEMISTRY, PERGAMON, OXFORD, GB, vol. 40, no. 6, 1 June 2008 (2008-06-01), pages 1404-1412, XP022679655 ISSN: 0038-0717 [retrieved on 2008-01-28] page 1409	5-9

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INTERNATIONAL SEARCH REPORT

International application No
PCT/IB2008/003484

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	PROME J C ET AL: "The pivotal role of tandem mass spectrometry in structural determinations of Nod factors produced by Rhizobia - Nod factors produced by wild-type strains of Mesorhizobium huakii and Rhizobium sp. mus10" INTERNATIONAL JOURNAL OF MASS SPECTROMETRY, ELSEVIER SCIENCE PUBLISHERS, AMSTERDAM, NL, vol. 219, no. 3, 1 September 2002 (2002-09-01), pages 703-716, XP004379440 ISSN: 1387-3806 page 706	5-9
X	D'HAEZE, WIM ET AL: "Nod factor structures, responses, and perception during initiation of nodule development" GLYCOBIOLOGY (2002), 12(6), 79R-105R CODEN: GLYCE3; ISSN: 0959-6658, 2002, XP002543414 cited in the application the whole document	5-9
X	DENARIE JEAN ET AL: "Rhizobium lipo-chitooligosaccharide nodulation factors: Signaling molecules mediating recognition and morphogenesis" ANNUAL REVIEW OF BIOCHEMISTRY, PALTO ALTO, CA, US, vol. 65, 1 July 1996 (1996-07-01), pages 503-535, XP009121807 ISSN: 0066-4154 the whole document	5-9